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THE MODE OF ACTION OF SULPHANILAMIDE. II. THE ANTISULPHANILAMIDE AND OTHER ANTI-BACTERIOSTATIC FACTORS IN BACTERIAL EXTRACTS.

H. N. GREEN AND F. BIELSCHOWSKY.

From the Department of Pathology, University of Sheffield.

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IN earlier work (Green, 1940) it was shown that bacteria survive in the presence of sulphanilamide (Su)* if the bacterial density reaches a "critical" level, a level which varies with the species or strain of bacteria and the concentration of Su. The greater the sensitivity of the bacteria to Su the greater is the "critical" density necessary for survival of the culture. This effect was shown to be due to the release from the bacteria of an anti-Su substance or substances. Active bacterial extracts were obtained from *Br. abortus* and other bacteria by different methods, and the anti-Su factor present in such extracts was called "P" (proliferation) factor, as the crude extracts (particularly those from *Br. abortus*) appeared to strongly stimulate the growth rate of many bacteria *in vitro*.

However, further purification of the *Br. abortus* preparation (Green and Bielschowsky, 1940) resulted in a fraction which, though its anti-Su potency was very great (2 μg. in 1 ml. of broth), had no effect on bacterial growth. The method used suggested that the bacterial anti-Su factor was an organic acid. Unfortunately owing to the high infectivity of the strain of *Br. abortus* used, further work on extracts of this organism was not possible. Work was continued with extracts from *Bact. coli* and later from *Staph. aureus*.

Meanwhile Woods (1940), whilst investigating the anti-Su activity of yeast extracts, made the important discovery that *p*-aminobenzoic acid (P.A.B.) and related compounds had an extremely powerful anti-Su action. Recently Rubbo and Gillespie (1940) have isolated *p*-aminobenzoic acid (P.A.B.) from yeast. Fildes (1940) and Woods (1940) suggested that P.A.B. is the bacterial anti-Su factor. Green and Bielschowsky (1940) were, however, unable to obtain the diazo reaction from their potent ether-soluble *Br. abortus* preparation. They also observed quantitative differences between the effects of P.A.B. and the bacterial extracts. They concluded that, whilst the properties of P.A.B. more closely resembled those of the bacterial factor than any of the other anti-Su factors described, they were not identical. Further work has however revealed that the ether-soluble bacterial anti-Su factor is, in all

* Su indicates *p*-aminobenzenesulphonamide.

probability, P.A.B. Attempts to explain differences in the action of our bacterial extracts and P.A.B. were of value, revealing the presence in crude extracts of other factors inhibitory to bacteriostatic substances. Also a comparison of these anti-bacteriostatic mechanisms on compounds related to Su has revealed facts bearing on current theories of the mode of action of Su.

The factor in bacterial extracts which accelerates bacterial growth will be described in a later paper. As, however, any factor which stimulates bacterial growth will act as a non-specific anti-Su substance (Green, 1940), it will be referred to, since its presence in the cruder bacterial extracts is responsible for some of the anti-Su activity observed in them. This factor will, in future, be called " P " factor.

For reasons of space it is impossible to give a detailed account of most of the experiments for which results are cited.

METHODS.

Assay of Anti-Sulphanilamide Activity.

The methods used for anti-Su factor assays were, in the main, similar to those previously described by Green (1940). The nutrient broth had the following composition: Lab-Lemco 20 g., peptone (Bacto-Difco) 30 g., NaCl 10 g., H_2O to 2 litres; pH 7·6. The results cited were all obtained in tests proceeding simultaneously in the same batch of nutrient media. Controls were set up for each experiment. All bacterial extracts were sterilized by boiling for 10 minutes.

The Brucella group, owing to their slow rate of growth, had been found useful in tests for both anti-Su and the growth-stimulating factors. In the present work a strain of *Br. paramelitensis* replaced *Br. abortus*.

Br. paramelitensis at first was less sensitive to Su than *Br. abortus*. With *Br. abortus* 1/10,000 Su was usually bactericidal to an inoculum of 200 per ml. of the medium, but was only bacteriostatic to a similar inoculum of *Br. paramelitensis*. In routine assays of anti-Su factor, 1/10,000 Su and inocula of 2 and 20 per ml. of medium were used. In the absence of anti-Su factor an inoculum of 20 usually showed turbidity in from 10 to 15 days, whilst an inoculum of 2 organisms only occasionally grew out, and then in from 15 to 25 days. For no determined reason the stock strain of *Br. paramelitensis* later became much more sensitive to Su.

In recent work we have used more rapid methods for determining the anti-Su potency of bacterial extracts, it having been first established that the preparation did contain the ether-soluble anti-Su factor. For this purpose a solution of 1/1000 Su in broth, with an inoculum of 4000 per ml. of medium of a stock strain of *Bact. coli*, has proved a useful combination. In the presence of optimal amounts of P.A.B. (a final concentration of 0·02–0·2 mg. per cent.) the first trace of turbidity appears in from 5 to 7 hours after the test solutions are placed in a 37° C. water-bath, concurrently with, or occasionally before, the appearance of turbidity in the control broth bottles. The control cultures in Su usually show turbidity in from 16 to 20 hours. It is thus possible by hourly observations to obtain a rough quantitative comparison of the anti-Su potency of test extracts in a period of 20 hours.

Sulphathiazole at a dilution of 1/1000 in nutrient broth inoculated with *Staph. aureus* was sometimes used in a parallel series. With an inoculum of 1000 turbidity appears in from 18 to 22 hours as compared with 7 to 9 hours in the presence of optimal amounts (20 mg. per cent.) of P.A.B., or in nutrient broth alone.

When sufficient material was available a triplicate series using *Br. paramelitensis*, *Bact. coli* and *Staph. aureus* was set up. This apparently elaborate method has often been justified by the results. As the action of the specific anti-Su factor is the same whatever the test organism, this method reduces the chance of a non-specific anti-Su effect due to a growth-promoting factor.

Latterly a Spekker absorptiometer has been used for estimating the degree of growth in bacterial cultures. The incubation period was chosen to give the majority of readings at the most sensitive point on the calibration drum, and before maximal growth had been reached in any of the cultures. This has been found an accurate method for determining the amount of anti-Su factor in terms of an equivalent amount of P.A.B.

Sources of Error.

Errors in experiments of this type may arise from the presence of growth-promoting or inhibiting substances in the preparation under test and from variations in the nutrient properties of the media. For this reason, in preliminary tests of all preparations, the experimental conditions have been such that the Su concentration was bactericidal to cultures in an optimal nutrient medium containing 1·5 per cent. peptone, which supported vigorous growth. Under such conditions the addition of more peptone or of serum has no significant influence on the outcome of the experiment. When, however, the conditions are such that Su produces only moderate bacteriostasis, it is found that additions to the medium, such as extra peptone, serum, tissue extracts and body fluids may produce an anti-Su effect. It may well be that some of these effects, also described by Lockwood (1940) and others, are due to the presence of traces of the specific anti-Su factor in the test materials used; that cannot be assumed unless a non-specific growth-promoting effect has been excluded by direct test.

More difficult to eliminate is the interference of " P " factor, which is present in many of the crude bacterial extracts we have studied. This factor appears to stimulate bacterial fission directly and therefore has a non-specific anti-Su effect even in an optimal nutrient medium. In its presence there is a significant and often considerable reduction in the " lag " period prior to the onset of the logarithmic phase in bacterial cultures. The effect has been observed using *Br. paramelitensis, Bact. coli* and *Staph. aureus*. Though a preparation from one bacterial species stimulates the growth of other species there appears (in contrast to the bacterial anti-Su factor) to be, in some instances, a much greater effect on the homologous species. The triplicate test (*v.s.*) is therefore of considerable value in deciding how far the anti-Su effect is due to the presence of " P " factor. " P " factor is effective in much lower concentrations of the crude bacterial extracts than is the anti-Su factor. By ether extraction at pH 4·5 the anti-Su factor can be obtained free of " P " factor. Boiling with *N* H_2SO_4 destroys much of the " P " factor, whilst the anti-Su factor is unaffected. Powerful growth-inhibiting substances in crude bacterial extracts frequently mask their true anti-Su potency. The inhibitory effect varies widely with the species and the mode of preparation of the extract. A method of removing many of the inhibiting substances has been developed (*v.i.*).

Preparation of Bacterial Extracts.

Br. abortus.

This organism provided the most potent of all our extracts, which gave, nevertheless, a completely negative diazo reaction. The method of extraction is summarized below and gives the results of the activity in 1/10,000 Su, using the method described by Green (1940).

	Anti-Su potency.	Minimal amount effective (μg. ml.).
Br. abortus (1·5–2 g. dry wt.) washed in distilled H_2O	*Nil*	—
Bacteria extracted twice with *N*/25 ammonia	++	—
Bacteria resuspended in 60 ml. H_2O, incubated 12 days at 37° C. Bacteria centrifuged down and supernatant Seitz filtered. Filtrate concentrated *in vacuo* to 10 ml. and 90 ml. alcohol added. Precipitate	*Nil*	—
Filtrate evaporated to dryness (10 mg. available)	++	33
Dissolved in 5 ml. H_2O and $Ba(OH)_2$ added. Precipitate (after Ba removed)	++	6
To the filtrate 9 volumes alcohol added. Precipitate	+	—
Filtrate (after removal of Ba) evaporated to dryness and residue dissolved in water	++	—
Solution acidified and extracted with ether:		
Ether-soluble material (diazo reaction negative)	++	2
Ether-insoluble material	*Nil*	—

Bact. coli.

A strain of *Bact. coli* was used as the standard source of the bacterial anti-Su factor. Its sensitivity to Su is relatively low (*v.s.*). As was indicated in a previous paper (Green, 1940) some organisms yield extracts rich in anti-Su factor by simply washing the organism with distilled

water. In the case of *Bact. coli* this method provides a crude extract containing much more solids, and therefore less amenable to chemical analysis, than those prepared from *Br. abortus* by Stamp's (1939) method, or by prolonged autolysis in H_2O. A second disadvantage was that coli extracts, both washings and autolysates, contain substances powerfully inhibitory to bacterial growth. The inhibitory substances can, however, now be largely eliminated (*v.i.*).

Coli washings (C_1).—24-hour agar cultures from 30 Roux bottles are washed off with about 300 ml. distilled H_2O. The suspension is filtered through muslin and centrifuged to remove the bulk of the bacteria. The supernatant fluid is Seitz filtered and the filtrate concentrated *in vacuo* at 40° C. to about 60 ml. Three volumes of absolute alcohol (higher concentrations of alcohol diminish the anti-Su activity of coli preparations) are then added and, after standing in the refrigerator overnight, the inactive precipitate is centrifuged down. The filtrate is concentrated nearly to dryness *in vacuo*, the residue dissolved in 30 ml. of H_2O and the insoluble material filtered off. This crude preparation is referred to in the text as C_1.

Separation of the organic acid fraction from C_1.—Preparation C_1 is brought to pH 4·5 with 2 *N* HCl and the solution is then extracted with peroxide-free ether in a Kutscher-Steudel apparatus for 20 hours. During the extraction the pH is maintained at 4·5 by adding a few drops of dilute HCl when necessary. After 20 hours no more active material can be extracted, though the watery phase still shows considerable anti-Su potency. The water-soluble fraction is now boiled with H_2SO_4 (final concentration 0·2 *N*) for 1 hour, the pH readjusted to 4·5 and ether extraction is repeated. A second, less potent ether-soluble fraction is obtained in this manner. The two ether solutions are pooled and shaken several times with 2 per cent. $NaHCO_3$. The active principle is taken up completely by the $NaHCO_3$ solution. Some of the growth-inhibiting material remains in the ether. The $NaHCO_3$ solution is acidified with dilute HCl to pH 4·4 and extracted for 9 hours with petroleum ether (B.P. 40°–60° C.). The petroleum ether removes most of the growth-inhibiting material (probably fatty acids) without loss of the anti-Su factor. This final fraction is referred to in the text as C_2. C_2 gives a strongly positive diazo reaction and the typical red colour of the Marshall (1937) reaction develops quickly. In the $NaHCO_3$ fraction, prior to the petrol ether extraction, some substance interfering with the diazo reaction is present, so that instead of a red, a brownish-red colour develops, and the result is quite inconclusive. The final volume of all fractions was adjusted so that 1 ml. was derived from the same weight of bacteria.

Coli autolysates.—The same method applied to *coli* autolysates (7 days at 37° C.) gives a fraction with similar properties to those of C_2, although in the crude autolysates the inhibitory substances completely mask the presence of the anti-Su factor.

Extracts from various types of bacteria.

Similar methods have been applied to *Br. paramelitensis*, *B. typhosus*, *Staph. aureus* and to an unclassified large Gram-negative coccus.* This latter organism appeared as a contaminant in media containing Su and P.A.B. It proved to be more sensitive to Su than any organism yet encountered; 1/10,000 Su was completely bactericidal to an enormous inoculum (3×10^{-9} per ml. of medium). All these organisms gave active extracts, but *Staph. aureus* appeared to be the best source of anti-Su factor.

ANTI-BACTERIOSTATIC FACTORS IN CRUDE *BACT. COLI* EXTRACTS. (C_1).

The crude *Bact. coli* extract (C_1) was antagonistic to some other bacteriostatic agents besides Su and related compounds containing a free *p*-amino group. Examples are given in Tables I and II.

The anti-bacteriostatic activity of C_1 against these four substances was not reduced by continuous ether-extraction at pH 4·5—a process which removed much of the anti-Su factor present. This ether-insoluble fraction contains the growth-stimulating "P" factor, and it is therefore possible that some of the anti-bacteriostatic effects described are similar to the effect of adding a larger inoculum to the test medium. The facts given, together with others to be considered in a later paper, suggest, however, that they are due to specific anti-bacteriostatic factors. If, as now seems likely, the specific bacterial

* Probably a member of the *Friedländer* group.

TABLE I.—*Inhibition of Bacteriostatic Action of Potassium Tellurite by an Extract* (C_1) *of* Bact. coli.

Inoculum: *Bact. coli*; 400 cells/ml. Volume of extract added: 0·5 ml. to 4·5 ml. broth. Final conc. potassium tellurite: 1/500,000.

Preparation added.	Viable count (organisms/ml.) after hours: 3	5	Turbidity after 24 hours.
None	15	0	None.
C_1: untreated	250	1,250	+
C_1: after acidification at pH 3	1,250	3,350	+
C_1: after acidification at pH 4	8,700	310,000	+++

(1) Potassium tellurite tested against *Bact. coli* and *Staph. aureus*. The addition of HCl to bring the pH of C_1 to 4·5 followed by refrigeration overnight and incubation for 1 hour at 37° C. increased the anti-tellurite effect very considerably. Similar treatment at pH 3 had little effect. No evidence was obtained that the increase in the anti-tellurite effect was due to an increase of the growth-stimulating properties of the extract.

TABLE II.—*Inhibition of the Bacteriostatic Action of* p-*Hydroxylaminobenzenesulphonamide,* p-*Nitrobenzenesulphonamide and Penicillin by an Extract* (C_1) *of* Bact. coli.

Test organism: *Staph. aureus*. 0·5 ml. of extract or P.A.B. (final conc. 20 mg. per cent.) added to 4·5 ml. medium.

Bacteriostatic agent.	Concentration.	Inoculum (cells/ml. medium).	Preparation added.	Turbidity after 18 hours.	
p-hydroxylaminobenzenesulphonamide	1/10,000	10	*Nil*	0	
		10	C_1	+++	
p-nitrobenzenesulphonamide	1/5,000	10	*Nil*	0	
		10	C_1	+++	
		10	C_2	trace	
		10	P.A.B.	,,	
				18 hours.	40 hours.
Penicillin	1/200,000	10^6	*Nil*	0	0
		10^6	C_1	0	++
	1/500,000	10^2	*Nil*	0	0
		10^2	C_1	0	+++

(2) *p*-hydroxylaminobenzenesulphonamide tested against *Staph. aureus* and *Bact. coli*. The effect was observed at bactericidal concentrations.

(3) *p*-nitrobenzenesulphonamide tested against *Br. paramelitensis*, *Bact. coli* and *Staph. aureus*.

(4) Penicillin* tested against *Staph. aureus* and *Strept. haemolyticus*. Penicillin was without effect on the growth of *Br. paramelitensis*. Complete inhibition was only obtained in slightly bacteriostatic dilutions. The effect was not due to the presence of active penicillase (Abraham and Chain, 1941), since the extracts were boiled prior to testing. *Staph. aureus* extracts did not appear to antagonize penicillin.

* A preparation kindly supplied by Prof. Florey.

anti-Su factor is an "essential metabolite," it might be expected that crude bacterial extracts would contain other "essential metabolites" to which the action of bacteriostatic agents, other than Su, are related.

P.A.B. was not antagonistic to any of the four substances, with the exception of *p*-nitrobenzenesulphonamide. In this case the inhibitory effect was very slight, and compared with that on *p*-aminobenzenesulphonamide was negligible. Miller (1941) has shown that sodium *p*-aminobenzoate is formed from sodium *p*-nitrobenzoate by *Strept. viridans*. It is probable that the effect of P.A.B. seen in our experiments was due to its antagonistic action on a small amount of *p*-aminobenzenesulphonamide formed by bacterial reduction of the *p*-nitro compound.

The extract C_2, containing the ether-soluble bacterial anti-Su factor, had a precisely similar action to P.A.B. on all four substances.

After hydrolysis and the second ether extraction (see "Methods") the watery phase still showed definite anti-Su activity. This residual activity is probably not entirely due to the presence of "P" factor, for much of this factor is apparently destroyed during hydrolysis. Recently Loomes, Hubbard and Neter (1941) have prepared fractions from yeast, which, after the extraction of P.A.B. and the destruction of the growth-promoting activity by acetylation and consecutive hydrolysis, still showed anti-Su activity. They conclude, therefore, that apart from the ether-soluble anti-Su factor in yeast, a second factor is present, which is not identical with the growth-promoting factor. Our own results with bacterial extracts could be interpreted similarly.

THE ORGANIC ACID FRACTION (C_2).

C_2 contains the ether-soluble, petroleum ether-insoluble organic acids and much of the anti-Su activity of the crude extracts. C_2 is amenable to exact quantitative assays, since most of the inhibitory and growth-promoting factors in the crude extract (C_1) are eliminated. It gives a strong diazo reaction, and quantitative colorimetric estimations in fairly good agreement with the results obtained by comparing its anti-Su activity with that of solutions of P.A.B. of known strength; e.g., a specially concentrated batch of C_2 which in bio-assay behaved like a 0·2 mg. per cent. P.A.B. solution, developed a colour of an intensity corresponding to 0·25 mg. per cent. P.A.B. (see Table III).

Whilst C_1 has a wide range of anti-bacteriostatic activities, C_2, like P.A.B., inhibits only Su, and derivatives with a free *p*-amino group (see following paper, Green and Bielschowsky, 1942). Our early work showed apparent differences in the properties of P.A.B. and those of bacterial extracts. The most important difference was that the diazo reaction was negative in the very potent organic acid fraction from *Br. abortus* (see "Methods"). Many differences in biological activity were observed, e.g. *4, 4'*-diaminodibenzene-disulphide inhibited the growth of the avian type of *Myco. tuberculosis*; in the presence of C_1 growth proceeded, whilst the addition of P.A.B. had no effect. It was later found that the bacterial extract (C_2) was also ineffective against the high disulphide concentration used. This effect of C_1 may be due to the non-specific anti-Su action of "P" factor, to a distinct ether-insoluble

TABLE III.—*Anti-sulphonamide Potency of the Bacterial Organic Acid Fraction (C_2) Compared with that of its Estimated Content of* p-*Aminobenzoic Acid.*

Extent of growth estimated by photoelectric method with six pooled cultures. Inoculum: *Bact. coli*, 4000 cells/ml. medium.

Drug.	Fraction C_2. Estimated final conc. P.A.B. in medium (mg. per cent.).	Fraction C_2. Per cent. incident light absorbed after 18 hours.	P.A.B. Final conc. P.A.B. in medium (mg. per cent.).	P.A.B. Per cent. incident light absorbed after 18 hours.
Sulphanilamide —(1/1000)	—	—	20·0	60
	—	—	2·0	75
	—	—	0·2	73
	0·025	67	0·025	70
	0·02	65	0·02	68
	0·005	44	0·005	46
	0·004	37	0·004	43
	0·002	23	0·002	29
	0·0002	7	0·0002	10
	Su control	9	Su control	7
	—	—	Broth control	77

Drug.	Fraction C_2. Estimated final conc. P.A.B. in medium (mg. per cent.).	Fraction C_2. Per cent. incident light absorbed after 40 hours.	P.A.B. Final conc. P.A.B. in medium (mg. per cent.).	P.A.B. Per cent. incident light absorbed after 18 hr.	P.A.B. Per cent. incident light absorbed after 40 hr.
Sulphathiazole —(1/1000)	—	—	20·0	77	94
	—	—	2·0	66	93
	—	—	0·2	0	76
	0·025	51	0·025	—	55
	0·02	48	0·02	—	54
	0·002	40	0·002	—	44
	0·0002	26	0·0002	—	33
	Suth. control	23	Suth. control	—	24
	—	—	Broth control	77	92

TABLE IV.—*Anti-Sulphanilamide Activities of* p-*Aminobenzoic Acid and a Bacterial Extract* on an Unclassified Gram-negative Coccus.*

Su concentration: 1/10,000; 0·5 ml. preparation + 4·5 ml. nutrient broth.

Preparation added.	Conc. (per cent.).	Time of appearance of turbidity (days) after inoculating with (cells/ml.). 10^7.	10^6.	10^5.	10^4.	10^3.	Remarks.
P.A.B.	0·2	1	1	2	∝	∝	10^5 sterile after 3 days.
,,	0·02	1	3	∝	∝	∝	10^6 ,, ,, 5 ,,
,,	0·002	1	∝	∝	∝	∝	10^7 ,, ,, 5 ,,
Bacterial extract	—	1	1	1	1	2	10^4 ,, ,, 4 ,,
							10^2 ,, ,, 3 ,,
None (control)	—	2	∝	∝	∝	∝	10^7 ,, ,, 4 ,,

* Obtained from a Su-resistant strain of *Br. paramelitensis* by method similar to that usedor preparation of C_1.

anti-Su factor, or to a specific anti-disulphide factor (see also Green and Bielschowsky, 1941).

Another interesting example of this difference in biological action was observed, using the Gram-negative coccus, which was so enormously sensitive to Su (see "Methods"). It was impossible to inhibit the bactericidal action of Su with any concentration of P.A.B. when the size of the inoculum fell to a certain level (Table IV). Crude bacterial extracts were effective below this level. It was later found that the bacterial fraction, C_2, behaved exactly like P.A.B. in similar anti-Su tests.

It is now clear that the properties of the crude bacterial extract (C_1) are due to the combined effect of ether-insoluble anti-bacteriostatic and growth-stimulating factors and the ether-soluble bacterial anti-Su factor. In a large number of experiments we have never observed any significant difference between the biological action of P.A.B. and that of C_2.

Chemical treatment of C_2 has also yielded results consonant with the theory that the specific anti-Su factor in bacteria is P.A.B.

(1) *Diazotization.*—The method described by Woods (1940) did not give satisfactory results. The higher concentrations of P.A.B. (0·02–0·2 per cent.) were found to be inactive when treated by Woods' method. On diluting such solutions, however, a reduced activity reappeared; bacterial extracts behaved likewise. The inactivity of the higher concentrations was probably due to the formation of a bacteriostatic substance. The incomplete inactivation of P.A.B. was shown to be due to an incomplete decomposition of the diazotized P.A.B. at 37° C. The method was modified by heating the diazotized solutions for 1 hour in a boiling water bath. This treatment completely inactivated both P.A.B. and C_2.

(2) *Oxidation with potassium permanganate and hydrogen peroxide.*—*N*/10 $KMnO_4$ was added until the pinkish colour persisted for 10 minutes (pH 9). The solution was kept overnight in the refrigerator, the MnO_2 removed, and the neutralized solution tested. H_2O_2 was added in a slight excess, the solution kept overnight in the refrigerator, and the excess was removed by boiling. Both methods of oxidation slightly reduced the anti-Su activity of C_2 and P.A.B.

(3) *Treatment with acid and alkali.*—There was no reduction in potency after boiling in *N* H_2SO_4 or *N* NaOH.

(4) *Adsorption and eluation.*—The bacterial anti-Su factor was adsorbed quantitatively by norit at pH 4–5. At pH 7 adsorption was incomplete and at pH 9 negligible. All attempts to eluate the anti-Su factor have given unsatisfactory results. The best yields were obtained by eluation with *N*/25 NaOH or NH_3.

(5) *Dialysis.*—The anti-Su factor in C_2 dialyses easily through "cellophane" membranes.

(6) *Precipitation by heavy metals.*—As found by Woods (1940) in the case of the yeast anti-Su factor, the bacterial factor is precipitated by mercuric acetate and incompletely by lead acetate, but not by phosphotungstic acid.

(7) *Colour reaction.*—The test described by Tauber and Laufer (1941) for P.A.B. was positive with C_2.

These tests indicate that the anti-Su factor in the bacterial fraction C_2 is P.A.B. or a closely related substance. Its isolation would involve the use of a relatively enormous weight of bacteria, for it is calculated that 30 Roux bottles (20 hours' growth) yield a C_2 fraction containing 20–40 μg. P.A.B.

BACTERIAL RESISTANCE TO SULPHANILAMIDE.

It was shown by Green (1940) that if *Br. abortus* survived in Su for some days a resistant strain developed, the resistance increasing progressively the longer the time of exposure. This resistance may be retained when the

organism infects the body (Green, 1941). Green (1940) gave some evidence that the wide differences in Su-sensitivity of different organisms were due to differences in the rate of diffusion of the anti-Su factor from the organism into the medium, rather than to differences in the rate of synthesis. Much further work has revealed that the problem is not simple, for biological assays of the anti-Su factor are inexact when made on relatively crude extracts. The most promising method is that of estimating the intensity of the diazo reaction in the organic acid fractions from the combined autolysates and washings of organisms differing greatly in sensitivity. Since we may not be able to do this in the near future, some other relevant observations are cited.

TABLE V.—*Comparison of the Anti-Sulphonamide Potency of Extracts from Stock and Su-Resistant Strains of* Br. paramelitensis.

Extracts prepared similarly to C_1, but from washings of *Br. paramelitensis*. 0·5 ml. preparation + 4·5 ml. nutrient broth.

Preparation added.	Time (in hours) of appearance of turbidity (average of four observations) with—		
	1/10,000 sulphanilamide. Inoculum *Br. paramelitensis*, 20 organisms/ml.	1/1000 sulphanilamide. Inoculum *Bact. coli*, 4000 organisms/ml.	1/1000 sulphathiazole. Inoculum *Staph. aureus* 1000 organisms/ml.
Extract from stock strain	70	10	16
Extract from Su-resistant strain	42	7	12
P.A.B. (20 mg. per cent.)	48	6	10
None (control)	408	18	22

The enormously Su-sensitive coccus (*v.s.*) might be expected to yield little or no anti-Su factor, yet the washings from it had a fairly potent anti-Su action. The washings from a Su-resistant strain of *Br. paramelitensis* obtained after culture of the organism in 1 : 1,000 Su for 20 days showed greater anti-Su activity than the washings of the stock strain from which the resistant strain developed (Table V). McLeod (1940) obtained comparable results in broth cultures of Su-fast and Su-sensitive pneumococci; the centrifuged bacterial cells, even after autolysis, showed no anti-Su activity. Both experiments show that there is a more rapid release of anti-Su factor from the Su-resistant than from the Su-sensitive strain, but neither prove that there is any difference in the amount produced. It should be noted also that autolysates from resistant strains of *Br. abortus* or *Br. paramelitensis* had no greater anti-Su activity than those from the corresponding stock strains. The results of many biological assays do not show a strict correlation between bacterial sensitivity to Su and the yield of anti-Su factor, so that a complete explanation of natural or acquired resistance must await further investigation. It seems likely that one of the following explanations for Su-resistance may prove correct: (1) A more rapid synthesis of P.A.B., (2) a more rapid release of P.A.B. from the organism into the surrounding medium, (3) a more rapid synthesis or release of P.A.B. by the organism occurring only in the presence of Su, or (4), a reduction in the P.A.B. requirements. Relating to (3), it was shown by Green (1940) that when *Br. abortus* is grown in broth containing Su,

the broth contains much more ether-soluble anti-Su factor than that from control cultures. A later experiment with 24-hour *Bact. coli* cultures showed a still more striking difference. It thus appears that Su may have a stimulating action on the rate of synthesis of P.A.B. by bacteria, to which possibly a Su-insensitive bacterium is able to respond more efficiently than a Su-sensitive one.

SULPHANILAMIDE AS A BACTERIAL GROWTH STIMULANT.

Observations suggesting that, under certain conditions, Su stimulated the growth of *Br. abortus* were made by Green (1940), and added confirmation to the findings of Finkelstone-Sayliss *et al.* (1937) working with *Strept. haemolyticus*. Further evidence has now been obtained.

TABLE VI.—*Growth-Promoting Effect of Sulphanilamide on* Staph. aureus.

Degree of growth estimated by photoelectric method in six pooled cultures. Inoculum : 100 cocci/ml. medium.

Conc. of sulphanilamide (g./ml.).	Conc. of P.A.B. (mg. per cent.).	Incident light absorbed after 10½ hrs.' growth (per cent.).
10^{-4}	0	12
4×10^{-5}	0	26
2×10^{-5}	0	39
10^{-5}	0	61
2×10^{-6}	0	56
10^{-6}	0	55
10^{-7}	0	54
10^{-3}	2	59
10^{-6}	2	59
0 (broth control)	0	23

Occasionally the stock strain of *Br. paramelitensis* failed to grow in the control medium at an optimum rate, or even, with small inocula, to grow at all. When this occurred it was often observed that the organism had grown normally in media containing both Su and P.A.B., although not in media containing Su alone. A similar effect was seen with *Staph. aureus*, particularly in primary cultures from stock (Table VI). This effect was observed when the bacteriostatic action of Su was inhibited by P.A.B. Direct tests revealed, however, that Su itself, at a non-bacteriostatic concentration, stimulated growth. The effect was more obvious when growth in control culture was suboptimal (Table VI).

It is doubtful whether growth-stimulation occurs when cultural conditions are optimal. Results were conflicting, and when a positive effect was obtained it was never so striking as that observed when growth in the control medium was subnormal.

One more significant fact should be mentioned. It was found that the growth of Su-resistant strains of *Strept. haemolyticus* and *Br. paramelitensis*

was stimulated in a concentration of Su which was bacteriostatic to the Su-sensitive strains. It seems that, within wide limits, the phenomenon is independent of the Su concentration so long as its bacteriostatic action is not exerted, i.e. in low concentration or when the action of higher concentrations is antagonized by added P.A.B. or by the anti-Su factors produced in the culture itself.

Related to Woods' (1940) and Fildes' (1940) theory of the mode of action of Su, these facts suggest that the bacterial enzyme for which P.A.B. is the normal substrate may be influenced by the presence of the Su molecule, with the result that a substance stimulating, or essential to, some phase of bacterial metabolism is produced. (We have found that a small but definite amount of Su disappears in the presence of a large concentration of *Bact. coli*.) This hypothesis would explain why stimulation is greatest when the bacterial growth-rate indicates that a substance essential in bacterial nutrition is not being produced in adequate amounts by the organism, or is deficient in the medium. It should be noted that P.A.B. itself is not growth-stimulating; at higher concentrations it is bacteriostatic. Whatever the explanation of these findings they provide another example of a substance which is depressant in high, and stimulating in low concentrations. The stimulant action of Su on plant growth (Grace, 1938) suggests that Su may have a wider biological significance.

DISCUSSION.

The bearing of this work on the mode of action of Su is discussed in a succeeding paper (Green and Bielschowsky, 1942). Here we are chiefly concerned with the nature of the ether-soluble bacterial anti-Su factor. Our results show that the chemical and biological properties of this factor are similar to those of P.A.B. and support Fildes' (1940) concept that P.A.B. is an essential metabolite. It seems probable that P.A.B. is utilized by all bacteria and synthesized by most, and it is an attractive hypothesis that the rate of its synthesis determines the degree of sensitivity of the bacterial cell to Su. This hypothesis is not yet proved, but it seems reasonably certain that sensitivity to Su is related to the rapidity with which P.A.B. passes from the bacterium into the medium. It may be of significance in this connection that a bacterium which was enormously sensitive to Su formed mucoid colonies and dense mucous-like material in broth culture. It is possible that a surface film of this substance prevents the rapid diffusion of P.A.B. out of the cell. At any rate, P.A.B. appeared to penetrate the bacterium slowly, for a very much higher concentration than is required by other organisms was needed to inhibit Su, in spite of the fact that there was no evidence that this coccus utilizes more P.A.B. than other bacteria.

Recent reports give proof of a vitamin-like action of P.A.B. Rubbo and Gillespie (1940) have identified P.A.B. with a growth factor for *Cl. acetobutylicum*. Ansbacher (1941) reports that P.A.B. is a "chromotrichia" factor for rats and cats and a growth-promoting factor for chicks. It must be assumed that P.A.B. being, according to Ansbacher, a member of the vitamin B complex, is very widely distributed and plays a part not only in the metabolism of bacteria and yeasts, but in that of the higher animals too. Woods

(1940) obtained extracts with some anti-Su activity not only from yeast but from ox muscle, liver and blood, urine, horse serum, hydrolyzed egg albumin and turnip. MacLeod (1940) obtained active extracts from casein after enzymic but not acid hydrolysis, and from muscle and other tissues. The activity of tissue extracts was greatly increased, and in the case of liver only present, after autolysis or acid hydrolysis. We, too, have obtained active ether-soluble fractions from urine, transplanted rat sarcomata and from some higher fungi, including the common mushroom. We have pointed out that the activity of very crude extracts is not necessarily due to P.A.B., since any substance improving bacterial nutrition will have a non-specific anti-Su action. Nevertheless in view of the widespread distribution of P.A.B., it now seems possible that the anti-Su action of some of these extracts is due to P.A.B. or a closely related substance. The chemical methods now available should soon provide the answer.

We have shown that some of the bacterial anti-Su factor is present in a conjugated form and only becomes ether-soluble after acid hydrolysis. The work of MacLeod (1940) indicates that the greater part of the anti-Su factor in tissues is in a conjugated form. We have not yet proved that the substance liberated by hydrolysis is actually P.A.B. although this is likely. Finally, it should be noted that after hydrolysis and ether extraction, the crude bacterial extract still has a definite, though much reduced anti-Su activity. This is not due to growth stimulation by " P " factor, most of which is destroyed by acid hydrolysis. It may be due to an ether-insoluble factor, a second bacterial anti-Su factor distinct from P.A.B.

SUMMARY.

(1) Proof is given that the ether-soluble bacterial anti-sulphanilamide factor is identical with, or closely related to *p*-aminobenzoic acid.

(2) The ether-insoluble fractions of crude bacterial extracts contain a factor (" P " factor) which has a non-specific anti-sulphanilamide action due to its growth-stimulating action on bacteria. They probably also contain a specific anti-sulphanilamide factor.

(3) The ether-insoluble fractions of crude bacterial extracts antagonize certain other bacteriostatic substances, against which the ether-soluble bacterial anti-sulphanilamide factor and *p*-aminobenzoic acid are ineffective. Only bacteriostatic substances which are antagonized by *p*-aminobenzoic acid have a true sulphanilamide-like mode of action.

(4) The factors determining bacterial resistance to sulphanilamide are discussed.

(5) Further evidence is given that under certain conditions sulphanilamide has a growth-stimulating action.

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REFERENCES.

ABRAHAM, E. P., AND CHAIN, E.—(1940) *Nature*, **146**, 837.
ANSBACHER, S.—(1941) *Science*, **93**, 164.
FILDES, P.—(1940) *Lancet*, i, 956.
FINKLESTONE-SAYLISS, H., PAINE, C. G., AND PATRICK, L. B.—(1937) *Ibid.*, ii, 792.
GRACE, N. H.—(1938) *Can. J. Research*, **166**, 143.
GREEN, H. N.—(1940) *Brit. J. exp. Path.*, **21**, 38.—(1941) *Brit. Med. J.*, i, 478.
Idem AND BIELSCHOWSKY, F.—(1940*a*) *Chem. Ind.*, **59**, 135.—(1940*b*) *Ibid.*, **59**, 850.—(1942) *Brit. J. exp. Path.* **23**, 13.
LOCKWOOD, J. S., AND LYNCH, H. M.—(1940) *J. Amer. Med. Ass.*, **114**, 935.
LOOMES, T. A., HUBBARD, R. S., AND NETER, E.—(1941) *Proc. Soc. exp. Biol. Med.*, **47**, 159.
MACLEOD, C. M.—(1940) *J. exp. Med.*, **72**, 217.
MARSHALL, E. K.—(1937) *J. biol. Chem.*, **122**, 263.
MILLER, J. K.—(1941) *J. Pharm. and exp. Ther.*, **71**, 14.
RUBBO, S. D., AND GILLESPIE, J. M.—(1940) *Nature*, **146**, 838.
STAMP, T. C.—(1939) *Lancet*, ii, 10.
TAUBER, H., AND LAUFER, ST.—(1941) *J. Amer. Chem. Soc.*, **63**, 1489.
WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **21**, 74.

THE MODE OF ACTION OF SULPHANILAMIDE. III. THE RELATION OF CHEMICAL STRUCTURE TO THE BACTERIOSTATIC ACTION OF AROMATIC SULPHUR, SELENIUM AND TELLURIUM COMPOUNDS.

H. N. GREEN AND F. BIELSCHOWSKY.

From the Department of Pathology, University of Sheffield.

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THE finding of Woods (1940) that *p*-amino benzoic acid (P.A.B.) inhibits the action of sulphanilamide (Su)* compounds, and the evidence that the bacterial ether-soluble anti-Su factor is probably identical with P.A.B. (Green and Bielschowsky, 1941), throws new light on the mode of action of Su, and certain hypotheses, which are not consistent with these experimental results, can be discarded.

The possibility that the Su effect is due to a transformation of Su into an "active" compound still exists however. The occurrence of a "lag" phase in the action of Su suggested that during this phase the "active" substance might be formed. Also the fact that some oxidation and reduction products of Su appear to be more potent than Su itself has led various investigators to suggest that one or other of these substances might be the "active" compound. Thus Mellon (1940) suggested that *p*-hydroxylaminobenzenesulphonamide might be formed under aërobic, and *p*-aminothiophenol under anaërobic conditions. It will be shown that many of these compounds can be excluded as "active" substances, since they are not inhibited by P.A.B. Since P.A.B. inhibits the action of Su *in vivo* as well as *in vitro*, any bacteriostatic substance

* Su indicates *p*-aminobenzenesulphonamide.

which is not so inhibited is unlikely to play any part in the biological action of Su. Therefore, by utilizing the anti-bacteriostatic effect of P.A.B. we were able to investigate the mode of action of some compounds which might possibly be formed from Su by bacteria.

In the present work the properties of a number of related sulphur, selenium and tellurium compounds are described and the bearing of these findings on current theories of the mode of action of Su is discussed. It should be explained why we tested various aromatic selenium and tellurium compounds. Joachimoglu (1926) found that the salts of selenious acid had a bactericidal action *in vitro*, particularly against the coli-typhoid group; the salts of tellurious acid had a still more pronounced action. It seems that S, Se and Te have a certain bacteriostatic activity in common, and we were interested to know if the aromatic *p*-amino compounds, in which S is replaced by Se or Te, were superior to the inorganic salts. Also it was thought that tests for a possible antagonistic action of P.A.B. and the bacterial anti-Su factor on these compounds might throw light on their mode of action.

METHODS.

Biological.

The bactericidal potency of the compounds was determined by inoculating the appropriate liquid media containing the compound under test. The technique was the same as that described by Green (1940) in similar tests on Su compounds.

Taking *p*-aminobenzenesulphonamide as the standard, the potency of the test substances may be expressed as the ratio of the number of bacteria killed by the same concentration or as the ratio of the minimal concentrations bactericidal to a standard number of bacteria. The first ratio, when the test substance has a significantly higher potency, obviously gives a much higher figure than the second.

A further method is a comparison between the rate of growth of a standard inoculum in similar concentrations of the test substances, the concentration being such that the more potent compounds exert only a bacteriostatic and not a bactericidal action. This is the most useful method, but except in the case of slow-growing organisms like those of the *Brucella* group, it requires hourly observations to detect the first appearance of turbidity, or one or more viable counts at fixed times. The method was used with *Br. paramelitensis* as test organism and later, with other organisms (chiefly *Bact. coli* and *Staph. aureus*), when a photo-electric method for determining the turbidity became available.

It is doubtful whether numerical expressions of the relative potencies of Su and related compounds have any absolute significance. Comparisons of the effective minimal concentrations are valueless when the size of the bacterial inoculum varies in different tests. Hypotheses still appear in the literature based on experiments in which the bacterial inoculum was not accurately standardized.

Chemical.

p-*aminothiophenol* was obtained according to the method of Hinsberg (1909).

4, 4′-*diaminodiphenyldisulphide* was obtained by air or H_2O_2 oxidation of *p*-aminothiophenol in alkaline solution and was recrystallized from dilute alcohol (M.P. 80° C.).

p-*nitrothiophenol* (M.P. 77° C.), 4, 4′-*dinitrodiphenyldisulphide* (M.P. 182° C.) and 4-*nitro*-4′-*aminodiphenylsulphide* (M.P. 142° C.) were all obtained by the method of Zincke (1913).

p-*nitrobenzenesulphonamide.*—4, 4′-dinitrodiphenyldisulphide was boiled under reflux in concentrated HNO_3 (sp. gr. 1·42) until most of the substance was dissolved. Then H_2O was added, the insoluble residue removed and the HNO_3 evaporated *in vacuo*. The *p*-nitrobenzoic acid thus obtained was converted into its NH_4-salt, which was treated with P_2Cl_5 and the resulting *p*-nitrobenzenesulphochloride converted into the amide by concentrated NH_4OH solution. (Crystallized from alcohol, M.P. 180° C.)

p-*hydroxylaminophenylsulphonamide* was prepared by reduction of *p*-nitrobenzenesulphonamide as described by Burton *et al.* (1940). The method of isolation was modified, since we were unable to obtain a pure substance by following their procedure. The alcohol was removed *in vacuo* at a low temperature (37° C.) whilst H_2 was passing through the solution. The insoluble material was separated by filtration of the aqueous residue and the filtrate placed in the refrigerator. The yield of crude *p*-phenylhydroxylaminosulphonamide was improved by adding NaCl. The substance separating out was dissolved in a small amount of warm water. In the evacuated desiccator the pure substance crystallized in needles when the solution was reduced to about half its original volume. During the later stages care was taken to minimize oxidation by working in an atmosphere of H_2. The yield was about 10 per cent. The melting point, like that of the products prepared by Bratton White and Marshall (1939) and by Thorpe and Williams (1941), was 140° C.

4, 4′-*diaminodiphenyldiselenide* was obtained according to Bauer (1913). The only modification was in the method of recrystallization of the crude product. As decomposition was observed in solutions at temperatures of around 80° C., the material was dissolved in warm alcohol (50° C.) and water added until definite turbidity appeared. In the refrigerator the diselenide crystallized in the form of yellowish needles of M.P. 81° C. Calculated for $C_{12}H_{12}N_2Se_2$; N, 8·19 per cent.: found; N, 8·25 per cent.

4, 4′ N-*acetyl-diaminodiphenyldiselenide* was obtained by acetylation of the above with acetic anhydride. Fine yellow needles were obtained from alcohol (M.P. 182° C.). Calculated for $C_{16}H_{16}O_2N_2Se_2$; N, 6·58 per cent.: found; N, 6·72 per cent.

N-*acetyl*-p-*aminophenylseleninic acid.*— *4, 4′-N*-acetyldiaminodiphenyldiselenide was treated with ice-cold concentrated HNO_3 (sp. gr. 1.42). The acid was slowly added until the evolution of gas ceased (*ca.* 3 ml. for 1 g. substance). Water was added and the crude acid filtered, using suction. The product crystallized in colourless needles from water after adding a few drops of alcohol and charcoal. (M.P. 178° C.). Calculated for $C_8H_9O_3NSe$; N, 5·58 per cent.: found; N, 5·66 per cent.

4, 4′-*dinitrodiphenyldiselenide.*—This substance was obtained by treatment of *p*-nitrophenylselenocyanide (Bauer, 1913) with NaOH or by reduction with Na_2S in alkaline solution. The *p*-nitrophenylselenocyanide was suspended in C_2H_5OH, 2 *N* NaOH added and the mixture heated to boiling point. A small amount of insoluble material was filtered off and the filtrate acidified with 2 *N* H_2SO_4. The amorphous precipitate was recrystallized twice from alcohol-acetone (1 : 1) after the addition of charcoal. (M.P. 175–176° C.). Calculated for $C_{12}H_8O_4N_2Se_2$; N, 6·97 per cent.: found; N, 7·22 per cent.

p-*nitrophenylseleninic* acid was obtained from the diselenide compound by a similar method to that described for the synthesis of *p*-aminophenylseleninic acid. It was recrystallized from alcohol after the addition of charcoal. (M.P. 212° C.). Calculated for $C_6H_5O_4NSe$; N, 5·99 per cent.: found; N 6·12 per cent.

4, 4′-*N-acetyldiaminodiphenylditelluride* was synthesized from acetanilide and $TeCl_4$ according to the method of Reichel and Kirschbaum (1936). The melting point of the substance (196–198° C. with decomposition) recrystallized either from glacial acetic acid or from CH_3OH was higher than stated by Reichel (172° C.). The compound crystallized in two different forms; from H_2O in fine red needles, as described by Reichel, and from absolute CH_3OH in large, flat yellow needles. On heating, the red crystals were converted into the yellow at 150° C. Calculated for $C_{16}H_{16}O_2N_2Te_2$; N, 5·35 per cent.: found; N, 5·25 per cent. Although Reichel was unable to hydrolyze the acetyl compound without decomposition, we found it possible. The amino compound was obtained by hydrolysis with alcoholic soda (1 g. substance in 50 ml. 4 *N* alcoholic NaOH was boiled under reflux for 2 hours) and extraction of the products with ether. From ether solution the *4, 4′*-diaminodiphenylditelluride was precipitated by 0·1/*N* H_2SO_4 or by 2 per cent. *p*-toluenesulphonic acid. The sulphate crystallized in dark red needles. It was fairly stable in the presence of traces of sulphuric acid, but when the excess of H_2SO_4 was removed by washing, decomposition started immediately. The *p*-toluenesulphonate (yellow needles) behaved similarly. Calculated for $C_{26}H_{28}O_6N_2Te_2$; N, 3·57 per cent., S, 8·18 per cent.: found; N, 3·92 per cent., S, 8·44 per cent. The free compound was extremely unstable. In the presence of water it decomposed immediately; in dry ether, decomposition started after 24 hours. It was thus impossible to use it for biological tests.

The microanalyses were made by Dr. Weiler and Dr. Strauss (Oxford).

AROMATIC SULPHUR COMPOUNDS RELATED TO SULPHANILAMIDE.

In the following section the term "oxidation" product is used for *p* hydroxylamino and *p*-nitro derivatives of Su; the term "reduction" product refers to compounds containing a *p*-amino group and bivalent S.

The theory, first advanced by Mayer and Oechslin (1937), that *p*-hydroxylaminobenzenesulphonamide was the "active" compound, was reconsidered by Burton *et al.* (1940), who compared the activity *in vitro* of the *p*-hydroxylamino and *p*-nitro compounds. They concluded that the *p*-hydroxylamino compound, or more probably some oxidation product intermediate between it and the *p*-nitro compound, is the "active" substance.

TABLE I.—*Bactericidal Potency of Various Aromatic Sulphur, Selenium and Tellurium Compounds against* Br. paramelitensis *and other Organisms.*

	Inoculum (organisms/ml. medium) failing to grow in the presence of test substances in a dilution of 1 in:				
	20,000.	50,000.	20,000.	20,000.	Inhibition by P.A.B.
Test organism.	*Br. paramelitensis.*		*Staph. aureus.*	*Bact. coli.*	
p-nitrobenzenesulphonamide	0	—	0 (B)	0 (B)	*nil* or ±
p-aminobenzenesulphonamide	2	0 (B)	0	0 (B)	++
p-hydroxylaminobenzenesulphonamide	40	0 (B)	0 (B)	0 (B)	*nil*
p-nitrobenzenesulphide	2×10^3	0 (B)	0 (B)	0	*nil*
p-aminobenzenesulphide	2×10^6	50	10^4	0 (B)	++
4, 4'-dinitrodibenzenedisulphide	2×10^7	2×10^2	10^2	0	*nil*
4, 4'-nitroaminodibenzenesulphide	10^3	2×10^4	—	—	*nil*
4, 4'-diaminodibenzenedisulphide	10^9	2×10^6	10^6	0 (B)	++
N-acetyl-*p*-aminobenzeneseleninic acid	0	—	—	0 (B)	—
p-nitrobenzeneseleninic acid	2×10^3	0 (B)	1–5	0 (B)	*nil*
4, 4'-N-acetyldiaminodibenzenetelluride	2×10^6	50	1–5	2×10^4	—
4, 4'-diaminodibenzenediselenide	2×10^9+	8×10^8	10^7	0 (B)	+
			50,000.	50,000.	
4, 4'-dinitrodibenzenediselenide	—	0	0 (B)	0	*nil*

(B) indicates significant bacteriostasis.
0 indicates that growth took place with an inoculum of 2 organisms (or less).

We have tested the bacteriostatic activity of a number of compounds which might be formed by the bacteria or their hosts (Table I). Organisms sensitive to Su were also sensitive to the other *p*-amino compounds tested, though the bactericidal potencies of these substances varied widely. However, in the case of the *p*-nitro compounds there was no such precise relationship. Some of the Su-sensitive organisms were more, and others were less, inhibited by them. For instance, *p*-nitrobenzenesulphonamide had only a very feeble bacteriostatic action against *Br. paramelitensis* at 1/10,000, a concentration at which the *p*-amino compound was bactericidal to the inoculum used (20 per ml.). *p*-Nitro and *4, 4'*-dinitrodibenzenedisulphide had no significant action on *Bact. coli*, whilst the corresponding *p*-amino compounds were bacteriostatic at equal concentrations. It may be noted here that whilst

4, 4'-diaminodibenzenediselenide was, of all the compounds prepared, the most effective against *Br. paramelitensis*, the corresponding dinitro compound was inactive. Burton *et al.* (1940) noted that *p*-nitrobenzenesulphonamide was less active on some organisms (e.g. *C. diphtheriae*, pneumococcus) than the *p*-amino compound, whilst on many organisms, and strikingly so on the anaërobes, the nitro compound was more active. We can confirm their finding that *p*-hydroxylaminobenzenesulphonamide is more active than the *p*-amino compound on organisms which are sensitive to Su, though the difference is often not as striking as one might expect if it were the actual " active " substance (see Table I). In fact its effectiveness against the three organisms tested was less than that of any of the aminobenzenesulphide compounds. If, indeed, the *in vitro* bactericidal potency of a derivative of Su gives any clue to the nature of the " active " substance, the " reduction " compounds (SH ; S-S) with an intact amino group should be considered before the " oxidation " compounds : *p*-aminobenzenesulphide was much more strongly bactericidal than *p*-nitrobenzenesulphide and this in turn than *p*-nitrobenzenesulphonamide.

The discovery of a specific anti-Su substance made possible a simple approach to the problem, for if the bacteriostatic action is not inhibited by P.A.B., or by the ether-soluble bacterial anti-Su factor, the substance is unlikely to be amongst the list of possible " active " compounds ; otherwise it would be necessary to make the unlikely assumption that the anti-Su action of P.A.B. was due to its preventing the oxidation of the *p*-amino group *in vitro* and *in vivo*. The effective ratio of molar conc. Su/molar conc. P.A.B., though varying with the bacterial species and the type and concentration of the Su compound, is high, and under the conditions used by Woods (1940) was from 5,000–25,000. This fact is not compatible with the above assumption that the -NH_2 group of P.A.B. rather than that of Su is preferentially oxidized by a bacterial enzyme. If Su is in fact oxidized into an " active " compound by a bacterial enzyme the affinity between the enzyme and Su must be very high if the effective bacteriostatic concentration of the oxidized compound, as shown by *in vitro* experiments, is to be reached. Moreover *p*-nitro benzoic acid should, on the basis of this theory, have no anti-Su action, whereas it was found to inhibit Su, though much less strongly than did P.A.B. The significance of this is increased by the finding that it was quite inactive against the corresponding *p*-nitrobenzenesulphonamide.

It was found (see Table I) that all the S and Se compounds with a *p*-amino group could be antagonized partially or completely by P.A.B. or the bacterial anti-Su factor (if a sufficiently high concentration of bacterial P.A.B. could be attained) ; the effective concentration of P.A.B. increased with the bactericidal potency of the compound, rising in this order : sulphanilamide, sulphapyridine, sulphathiazole and the sulphide compounds. The -NHOH compound was not antagonized by P.A.B., and the -NO_2 compounds were unaffected or affected so slightly that there could be no question of a specific inhibition by P.A.B.

All the above facts suggest that the bacteriostatic actions of *p*-hydroxylamino and *p*-nitrosulphonamide compounds have mechanisms different from the *p*-amino compounds.

THE BIOLOGICAL ACTION OF 4, 4′-DIAMINODIBENZENEDISULPHIDE.

Any compound having claims to be the " active " compound should have all the following properties : (1) Act without delay ; (2) inhibit the growth of all Su-sensitive bacteria at a lower concentration than Su ; (3) show a similar relationship between the size of the inoculum and the effective bactericidal concentration as do the Su compounds, i.e. the ratio, effective bactericidal concentration/size of inoculum should rise steeply with increasing dilution of the compound (see Green, 1940) ; (4) be inhibited by P.A.B.

Of the substances tested, *4, 4′*-diaminodibenzenedisulphide complied best with these conditions, though the related compound, *p*-aminothiophenol, was, except for a weaker bactericidal effect, similar in action.

(1) The disulphide compound in a bactericidal concentration (1/50,000) completely killed an inoculum of *Br. paramelitensis* (2×10^3 per ml. of medium) in less than 1 hour, and an inoculum of 2×10^6 in 1–2 hours. By contrast, a solution of 1/10,000 Su inoculated with 2–5 organisms per ml. was not sterile until about 48 hours after implantation. The disulphide compound (1/10,000) killed an inoculum of 2×10^4 per ml. *Staph. aureus* almost immediately and one of 2×10^6 in less than 30 minutes.

It should be emphasized that with a constant inoculum the sterilization time in a broth solution of disulphide diminishes (as is the case with Su) as the concentration rises, and, with the same concentration, rises as the inoculum increases. When, however, the concentration is well above the minimal bactericidal level, the size of the inoculum, within wide limits, makes little difference, as sterilization occurs almost immediately.

It can be concluded that the disulphide compound acts much more rapidly than, and without the " lag " period observed with Su compounds.

(2) *4, 4′*-diaminodibenzenedisulphide was bactericidal to Su-sensitive organisms (*Br. abortus, Br. paramelitensis, B. anthracis*) at a much lower concentration than was any member of the Su group. This is shown for *Br. paramelitensis* in Table II. A definite bacteriostatic effect on both *Br. paramelitensis* and *Staph. aureus* was observed, even at a $1/10^8$ dilution, the highest used. Against the highly Su-sensitive coccus previously referred to (Green and Bielschowsky, 1941) it was enormously active, being bactericidal at 1/500,000 to an inoculum of 200 per ml., and strongly bacteriostatic at a dilution of $1/10^9$.

Against the group of bacteria relatively insensitive to Su, the disulphide compound proved in general more effective than Su. This is well shown in the case of *Staph. aureus* (Table I). Against *Bact. coli*, however, it was not as effective as might have been expected. At a dilution of 1/10,000 it was mildly bacteriostatic to a small inoculum, but only slightly more so than 1/10,000 Su. *Bact. coli* was much more sensitive to the Su compounds than was *Staph. aureus*, but with the disulphide compound the relationship was reversed, e.g. sulphathiazole was much more inhibitory to *Staph. aureus*, and very much more to *Bact. coli*, than was sulphanilamide, whereas *4, 4′*-diaminodibenzenedisulphide was very much more inhibitory to *Staph. aureus* and much less to *Bact. coli* than was sulphathiazole. This was one of the significant differences between the action of the disulphide compound and that of sulphathiazole.

The most striking difference was, however, that the presence of blood, plasma or serum even at a level as low as 5 per cent. inhibited the bactericidal action of the disulphide compound on all organisms tested. Under these conditions it had only a mild bacteriostatic action, even on bacteria highly sensitive to Su.

(3) The disulphide compound showed the same relationship between concentration and number of bacteria killed as was previously shown by Green (1940) in the case of Su. The ratio, Su concentration : size of inoculum killed, rose rapidly as the Su concentration fell. This effect, which is typical of the action of Su, was observed in tests with various organisms.

(4) It has already been noted that P.A.B. partially inhibits the action of the disulphide and related compounds. The quantitative relationship between the two substances is of possible significance in relation to the mode of action of this compound. If, as Woods (1940) postulated, the action of Su is due to a blocking by Su of the enzyme concerned in the further utilization of P.A.B. in the bacterium, the more potent the compound, the greater should be the molar concentration of P.A.B. required for inhibition. Woods (1940) found that the molar conc. sulphonamide : molar conc. P.A.B. ratios for Su and sulphapyridine were roughly 5000 and 25,000. We found that sulphathiazole required a still greater concentration of P.A.B. ; in one experiment, using 1/1000 sulphonamide and *Bact. coli*, the ratio was roughly 500 for sulphathiazole and 25,000 for Su. Woods (1940) also showed that the molar conc. Su : molar conc. P.A.B. ratio remained constant at different Su concentrations. We have confirmed this, but with the qualification that the ratio is only constant if the range of Su concentrations used are at bactericidal or strongly bacteriostatic levels for the test organism. Below these levels the Su : P.A.B. ratio tends to rise, and with relatively insensitive organisms, such as *Staph. aureus* or *Bact. coli*, rises rapidly. These variations are probably due to the supplementing of the added P.A.B. by that supplied by the bacteria. The rapid action of the disulphide compound should prevent any significant difference in the amount of bacterial P.A.B. produced. It was found, however, that at strongly bactericidal concentrations of the drug it was impossible to inhibit its action with any concentration of P.A.B. (see Tables II and III). If the theory of competitive inhibition of an enzyme is correct, this result would suggest that the disulphide compound has a greater affinity for the enzyme concerned than has P.A.B.

This theory was tested by observing the effect of diminishing concentrations of P.A.B. against disulphide in a concentration which was strongly bacteriostatic to the larger inocula used. It will be seen (Table II) that at a concentration of 2 mg. per cent. it required 2–5 mg. per cent. P.A.B. to produce complete inhibition of the bacteriostatic effect. The ratio molar conc. disulphide : molar conc. P.A.B. is thus approximately between 0·2 and 0·5. This fact may explain why the anti-disulphide effect of P.A.B. was not observed at disulphide concentrations above a certain level—a level which was found to fall as the sensitivity of the test organism increased. We have found that P.A.B. itself is slightly inhibitory to bacterial growth at a concentration of and above 5 mg. per cent. (1/20,000). Assuming that the ratio molar conc. disulphide : molar conc. P.A.B. is between 0·5 and 0·2, it can be estimated that P.A.B.

should have a much diminished anti-disulphide effect at disulphide concentrations of between 1/50,000 and 1/20,000. This was found to be the case. In Table III it will be noted that the antagonistic effect of P.A.B. progressively diminishes as the disulphide concentration rises until at a dilution of between 1/30,000 and 1/20,000 it ceases completely. In the case of the much more sensitive *Br. paramelitensis* P.A.B. had no antagonistic action at a disulphide concentration greater than 2 mg. per cent. (1/50,000), and even at this level only when a fairly large inoculum was used (see Table II).

TABLE II.—*Inhibitory Action of* p-*Aminobenzoic Acid on* 4, 4′-*Diaminodibenzenedisulphide.*

Test organism : *Br. paramelitensis.* Disulphide concentration : 1/50,000.

P.A.B. (mg. per cent.).	Incident light absorbed (per cent.) 48 hours after inoculation with (organisms/ml. medium) :			
	2×10^8.	2×10^7.	2×10^6.	2×10^5.
0	37	17	0	0
200·0	28	0	0	0
20·0	57	51	*57	0
5·0	65	67	58	0
2·0	66	65	57	0
1·0	62	51	29	0
0·2	50	27	17	0
0·02	43	15	14	0
Control (broth alone)	62	66	35	29

* Note apparent growth stimulation.

TABLE III.—*Inhibitory Effect of* p-*Aminobenzoic Acid, Bacterial Anti-Sulphanilamide Factor* (C_2), *and Crude* Bact. coli *Extract* (C_1) *on* 4, 4′-*Diaminodibenzenedisulphide.*

Inoculum : *Staph. aureus,* 10^4 cocci/ml. medium.
C_1 : The addition was 10 per cent. (by volume) of medium.
C_2 : The addition gave an estimated final concentration of P.A.B. in the medium of 0·025 mg. per cent.

Inhibitor added.	Mg. per cent.	Incident light absorbed (per cent.) after 16 hours' growth in a dilution of 4, 4′-diaminodibenzene disulphide of 1 in—				
		20,000.	30,000.	40,000	50,000.	100,000.
None	—	0	7	45	44	68
P.A.B.	20·0	0	26	54	49	72
,,	2·0	0	22	59	59	78
,,	0·2	0	17	37	49	73
,,	0·02	0	7	35	45	70
C_2	—	0	5	46	46	69
C_1	—	25	44	60	65	83
Control (broth alone)	—	71	80	80	75	80

The facts show that the ratio molar conc. disulphide : molar conc. P.A.B. may be less than unity. It is difficult, however, to account for the high bactericidal activity of the disulphide compound solely on the hypothesis that it competes more effectively than does P.A.B. for the enzyme concerned. On this hypothesis it ought to be much more effective than Su against all bacteria, whereas it was only slightly more effective against certain Gram-negative bacteria, e.g. *Bact. coli*, *Ps. pyocyaneus*, and indeed much less effective than was sulphathiazole. It will be noted in Table III that a crude extract (C_1) from *Bact. coli* partly antagonized the disulphide compound when P.A.B., and the bacterial fraction (C_2) containing P.A.B., were ineffective, and that it had a more powerful anti-disulphide action than P.A.B. This action of the crude bacterial extract may be due to the presence of the growth-promoting " P " factor, to a distinct ether-insoluble anti-Su factor or to a specific anti-disulphide factor. Whatever the explanation, the facts suggest that the mode of action of the disulphide compound, though closely related to that of Su, may have another which supplements its Su-like action.

Two more points of resemblance between Su and the disulphide compound were noted. Firstly, Su-resistant strains which had developed from Su-sensitive bacteria grew well in concentrations of the disulphide compound which were lethal to the sensitive strain. Acquired resistance to Su is evidently associated with resistance to the " reduction " compounds. Secondly, in very high dilutions ($1/10^7$–$1/10^9$) of disulphide, growth stimulation, under conditions in which stimulation was seen with Su itself (see Green and Bielschowsky, 1942) was observed. It is concluded that the mode of action of the " reduction " products of Su bears some relation to that of the Su compounds.

AROMATIC SELENIUM AND TELLURIUM COMPOUNDS.

Some Se and Te compounds related to the aromatic S compounds already discussed were prepared (see " Methods ") and tested. Of these *4, 4'*-diaminodibenzenediselenide proved the most potent; in other respects its antibacterial properties closely resembled those of the corresponding disulphide compound (see Table I). It was strongly bactericidal to all Su-sensitive bacteria, and had a higher activity against *Staph. aureus* than any of the *p*-amino aromatic compounds tested. It was bactericidal to a small inoculum (200 per ml. of medium) of *Br. paramelitensis* at a dilution of 1/300,000, whilst the inorganic salt, sodium selenite, was inactive at a concentration containing an amount of Se equal to that in a 1/20,000 solution of the diselenide compound. The corresponding *p*-nitro compound at a dilution of 1/50,000 was inactive against *Br. paramelitensis* but was mildly bacteriostatic to *Staph. aureus*. Owing to its insolubility accurate tests could not be made at higher concencentrations. It should be noted, however, that at a dilution of 1/20,000 *p*-nitrobenzeneseleninic acid had a significant bactericidal effect on all these organisms. Unfortunately we were unable to prepare *p*-aminoseleninic and selenonic compounds of sufficient purity as they are very unstable. The corresponding acetyl compounds had no very significant anti-bacterial action.

Still more difficult to handle are the corresponding Te compounds, the only known compound being *4, 4'-N*-acetyldiaminodibenzeneditelluride. This compound was very difficult to dissolve, and the highest concentration which

could be tested was 1/20,000. At this concentration it was moderately bactericidal to *Br. paramelitensis* and *Bact. coli*, and slightly so to *Staph. aureus* (see Table I). In the inorganic form, as potassium tellurite, Te was much more bactericidal to *Bact. coli*, a 1/200,000 solution being as effective as 1/20,000 ditelluride. The inorganic compound was also more effective against *Staph. aureus* when equimolecular amounts of Te were compared, but against the Su-sensitive organism, *Br. paramelitensis*, its action was very weak compared with that of the organic compound.

In striking contrast to the activity of the related Te compound, both *p*-acetylbenzenesulphonamide and *4, 4'-N*-acetyldiaminodibenzenediselenide were only feebly bacteriostatic. The greater effect of the related Te compound may be significant. The evidence suggests that, in aromatic compounds containing an amino group in the *para* position, the substitution of S by Se and by Te causes a progressive increase in the bactericidal potency.

It is of particular interest that the only one of these Se and Te compounds which had a free *p*-amino group (*4, 4'*-diaminodibenzenediselenide) was partially antagonized by P.A.B. Exact determinations of the ratio molar conc. diselenide : molar conc. P.A.B. were not made, but the ratio was less than that of the corresponding disulphide compound. It was, however, not possible to produce an optimal growth-rate in a bacteriostatic concentration of the diselenide compound by adding P.A.B.

The activity of diselenide was reduced in the presence of blood plasma, but to a lesser degree than that of the corresponding disulphide compound. Moreover, unlike disulphide, preliminary *in vivo* tests suggest that when applied locally it has a strong bactericidal effect in the tissues.

THE RELATION BETWEEN CHEMICAL STRUCTURE AND BACTERIOSTATIC ACTION.

Fourneau *et al.* (1937, 1938) suggested that the active principle of the group of Su compounds is the N⟨ ⟩S radical. We consider that this hypothesis is fundamentally correct, although it has been refuted on the basis of the bacteriostatic action of *p*-nitrobenzoic acid (Mayer and Oechslin, 1939) and that of dihydroxydiphenylsulphone (Levaditi *et al.*, 1938). Rosenthal *et al.* (1939) studied the same nitro compounds and a number of aromatic arsenic compounds ; of these *4*-nitroaminodiphenylarsenic acid, the corresponding arsine oxide and arsine were active *in vivo* against streptococci. Acetylation caused an increase in their potency ; the symmetrical *4, 4'*-amino compounds were inactive. This work has not necessarily any bearing on sulphanilamide action, since the substitution of S by As may produce an entirely new type of chemotherapeutic compound. Their own results suggest this, for in view of the inactivity of the diaminodiphenylarsenic compound, it is obvious that the bacteriostatic effect of the symmetrical nitro-amino compounds is partly due to the nitro group. It is probably therefore of a different nature from that of the Su grouping which, moreover, as we have confirmed, loses most of its activity after acetylation. The action of *p*-nitrobenzoic acid is, on the other hand, relatively feeble, and the types of bacteria inhibited are not identical with those on which Su acts.

As we have shown, the antagonistic effect of P.A.B. is lacking, or only very slight, when the *p*-NH_2 compound is oxidized to the corresponding -NHOH or -NO_2 compound. All these facts suggest that the mode of action of organic nitro compounds is not identical with that of the *p*-aminophenyl sulphur compounds. Our results go to prove that the oxidation of *p*-aminobenzenesulphonamide gives a compound of different properties which may have an increased or decreased bacteriostatic action, according to the type of bacteria used. We suggest a modification of Fourneau's " active radical," preferring the grouping NH_2 S to N S, since our evidence suggests that the *p*-amino group is one of the essential features of Su activity.

One can draw a certain parallel between organic compounds containing the elements of the 5th B group (As, Sb and Bi) with their striking trypanocidal and spirocidal activity and compounds containing the elements of the other B group (S, Se and Te) with their bacteriostatic activity. This parallelism may be extended further, for just as the trypanocidal arsenic compounds containing trivalent As are more active than those containing pentavalent As, so the divalent S compounds tested by us were more active *in vitro* than the hexavalent. It seems of interest also that so many active compounds in both the 5th and 6th groups have in common an amino group in a *para* position to the elements of these groups.

Shinn (1939) suggested that the sulphanilamide radical in itself has no particular significance, but that its action may be due to its influence on the amino group. Our own results suggest that there is a certain relationship between the stability of the compound and its anti-bacterial action. Firstly, the *p*-amino compounds are in general more unstable than the inactive *o*- and *m*- compounds. Secondly, the diselenide is more unstable and more active than the disulphide compound, which in its turn is less stable but more active than sulphanilamide itself. Finally, the inactive or feebly active acetyl compounds are more stable than those with a free *p*-amino group. We conclude provisionally, therefore, that the influence exerted by the *p*-amino group on the S or Se molecule or *vice-versa* is of fundamental importance in determining the bacteriostatic action of these compounds.

DISCUSSION.

p-hydroxylaminobenzenesulphonamide has been regarded by several investigators as being actually responsible for the bacteriostatic action of Su. The -NHOH compound is a strong inhibitor of catalase. It was shown by Main *et al.* (1938) that ultra-violet irradiation of Su in water produced solutions with high anti-catalase activity, and it was assumed that the -NHOH compound was formed. Benoy (1940) has shown however that the ultra-violet effect is not specific, since irradiated benzene or benzenesulphonamide or sodium benzenesulphonate similarly inhibit catalase. Mellon *et al.* (1940) have assumed that the hydroxylamino-compound, by inhibiting catalase, produces an accumulation of H_2O_2 which kills the bacteria. As however Avery and Neill (1924) showed that peroxide production by pneumococci can proceed when conditions do not permit of active proliferation, the argument seems weak. Finally it should be mentioned that none of the claims to have identified hydroxylamine

compounds in the urine after the administration of Su have been confirmed. It must be concluded that the evidence for the H_2O_2 theory is insufficient. Our results also show that the hydroxylamino-compound has not the properties of an " active " compound, since it is not antagonized by P.A.B.

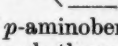
We have already shown that the " reduction " products of Su have more claim than the " oxidation " products to be considered as " active " substances. The important point is that they are antagonized, under certain conditions, by P.A.B., whilst the " oxidation " products are not. It is not claimed, however, that *4, 4'*-diaminodibenzenedisulphide is the actual compound responsible for the biological action of Su. The very striking inhibition of this compound by small amounts of blood plasma and its slight, if any, therapeutic action make it unlikely that it plays any part in the action of Su *in vivo*. It is nevertheless of interest that a substance related to Su exists which, in all other respects, complies with the requirements of an " active " compound. The concept of an " active " compound is not necessary for a proper interpretation of the mode of action of Su.

There are reasons for rejecting all the theories of Su action except the one first advanced by Fildes (1940) and Woods (1940) that the bacterial enzyme reaction involved in the further utilization of *p*-aminobenzoic acid is subject to competitive inhibition by Su. No result in our investigations conflicts with this concept. In the earlier part of our work we were not inclined to accept the theory in its entirety because the anti-Su action of the crude bacterial extracts appeared to differ from that of P.A.B. in some respects. It has now been shown by Green and Bielschowsky (1941) that the properties of the ether-soluble bacterial anti-Su factor and P.A.B. are indistinguishable. Our results also give strong support to the theory that Su inhibits the enzyme reaction involved in the further utilization of P.A.B., and not that involved in the synthesis of P.A.B. itself.

It seems to us that Su, and compounds closely related to it, possess their unique qualities because they are able to block this essential bacterial enzyme reaction, and are also sufficiently stable to remain unchanged in the tissues for some time. There seems to be no valid reason for the hypothesis that Su itself is inactive, and that its activity is due to a product formed from it by bacterial or tissue agencies. The varying potencies of the well-known compounds related to Su can be explained by the additional effect of the heterocyclic half of the molecule of these compounds on other metabolic processes, peculiar to, or more important to, the particular bacterium concerned.

No bacterium, of a wide range tested, proved completely insensitive to Su in sufficient concentration. If the above theory is correct, the metabolic process concerned in the utilization of P.A.B. must be common to all bacteria. It would follow that the degree of sensitivity to Su is determined either by the degree to which this process is essential to the life-cycle of the bacterium, or to the rate at which P.A.B. is synthesized and/or released into the surrounding medium. The next advance in bacterial chemotherapy may be the discovery of a substance which will react with P.A.B. *in vivo* or reduce the rate of synthesis of P.A.B. by bacteria, without seriously affecting the tissue cells. Such a hypothetical substance in combination with Su might well inhibit the growth of all types of bacteria.

SUMMARY.

(1) On the basis of *in vitro* findings with a number of aromatic sulphur compounds, it is suggested that the active principle of compounds with a sulphanilamide-like action is the NH_2  S radical.

(2) The antagonistic action of *p*-aminobenzoic acid on sulphanilamide compounds was utilized to determine whether certain " oxidation " and " reduction " products of sulphanilamide have a mode of action resembling that of sulphanilamide.

(3) Compounds containing an oxidized *p*-amino group, e.g. *p*-hydroxylaminobenzenesulphonamide and *p*-nitrobenzenesulphonamide, are not antagonized by *p*-aminobenzoic acid.

(4) Compounds containing a *p*-amino group and bivalent sulphur are antagonized by *p*-aminobenzoic acid, but after oxidation of their amino groups, *p*-aminobenzoic acid is ineffective. They have a much stronger bacteriostatic action on many bacteria than has sulphanilamide. They appear to have a rather similar mode of action to that of sulphanilamide, but differ in losing much of their activity in the presence of serum.

(5) The anti-bacterial properties of the most potent compound, *4, 4'*-diaminodibenzenedisulphide, are described in detail. Though they comply best with those of a postulated " active " compound, it is considered that the mechanism of action of sulphanilamide can be explained without assuming that an " active " compound is formed.

(6) The findings suggest that the more effectively a compound blocks the bacterial enzyme whose natural substrate is *p*-aminobenzoic acid, the greater is its bacteriostatic action.

(7) The sensitivity of bacteria to sulphanilamide is probably determined by the rate at which *p*-aminobenzoic acid is synthesized in the bacterial cell and/or is released into the surrounding medium.

(8) Various hypotheses of the mode of action of sulphanilamide are discussed and reasons for rejecting them given. On the basis of our results, the hypothesis advanced by Fildes and Woods is considered to provide the best explanation of the facts.

(9) Tests with several aromatic selenium and tellurium compounds suggest that the substitution of sulphur by selenium or tellurium results in compounds whose bacteriostatic properties are related to those of the corresponding sulphur compounds. Some evidence was obtained that the substitution of sulphur by selenium and by tellurium causes a progressive increase in the anti-bacterial potency of these compounds.

(10) Other aspects of the relationship between chemical structure and bacteriostatic action are discussed.

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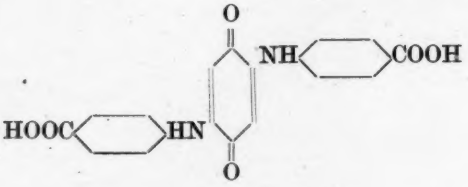
Addendum.

Oettel (1936) observed greying of the fur in cats during chronic hydroquinone poisoning. Martin and Ansbacher (1941) reported that the colour of

the hair could be restored or the change prevented by the addition of P.A.B. to the diet. We therefore tested whether the bacteriostatic effect of hydroquinone and its oxidation product, benzoquinone, could be antagonized by P.A.B. After trial tests conditions were found in which such an antagonism could be demonstrated. The best explanation of this effect is probably that P.A.B. in H_2O at 37° C. reacts immediately with quinone with the formation of—



This substance was synthesized and the sodium salt tested. It showed no anti-Su action and tested alone was weakly bacteriostatic.

REFERENCES.

AVERY, O. T., AND NEILL, J. H.—(1924) *J. exp. Med.*, **39**, 347, 357, 543.
BAUER, H.—(1913) *Ber. dtsch. chem. Ges.*, **46**, 92.
BENOY, M. P.—(1940) *J. Franklin Inst.*, **230**, 402.
BRATTON, A. C., WHITE, H. J., AND MARSHALL, E. K. (jun.).—(1939) *Proc. Soc. exp. Biol. and Med.*, **42**, 487.
BURTON, H., MCLEOD, J. W., MCLEOD, T. S., AND MAYR-HARTING, A.—(1940) *Brit. J. exp. Path.*, **21**, 288.
FILDES, P.—(1940) *Lancet*, i, 956.
FOURNEAU, E., TREFOUEL, J., NITTI, F., AND BOVET, D.—(1937) *Bull. Acad. Med.*, **118**, 210.—(1938) *C. R. Soc. Biol., Paris*, **127**, 393.
GREEN, H. N.—(1940) *Brit. J. exp. Path.*, **21**, 38.
Idem AND BIELSCHOWSKY, F.—(1940) *Chem. Ind.*, **59**, 135.—(1942) *Brit. J. exp. Path.*, **23**, 1.
HINSBERG, O.—(1909) *Ber. dtsch. chem. Ges.*, **42**, 3367.
JOACHIMOGLU, G.—(1926) *Biochem. Z.*, **107**, 300.
LEVADITI, C., GIRARD, A., AND VAISMAN, A.—(1938) *C. R. Soc. Biol., Paris*, **127**, 19.
MAIN, E. R., SHINN, L. E., AND MELLON, R. R.—(1938) *Proc. Soc. exp. Biol. Med.*, **39**, 310.
MARTIN, G., AND ANSBACHER, S.—(1941) *J. biol. Chem.*, **138**, 441.
MAYER, R. L., AND OECHSLIN, O.—(1937) *C. R. Soc. Biol., Paris*, **125**, 181.—(1939) *C. R. Soc. Biol., Paris*, **130**, 211.
MELLON, R. R.—(1940) *Amer. J. Med. Sci.*, **199**, 749.
OETTEL, H.—(1936) *Arch. exp. Path. Pharm.*, **183**, 319.
REICHEL, L., AND KIRSCHBAUM, E.—(1936) *Liebigs Ann.*, **523**, 211.
ROSENTHAL, S. M., BAUER, H., AND ELVOVE, E.—(1939) *Pub. Health Rep.*, **54**, 1317.
SHINN, L. E.—(1939) *J. Amer. Med. Ass.*, **113**, 1714.
THORPE, W. V., AND WILLIAMS, R. T.—(1941) *Biochem. J.*, **35**, 61.
WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **21**, 74.
ZINCKE, T.—(1913) *Liebigs Ann.*, **400**, 2.

THE INACTIVATION OF VACCINIA VIRUS BY RADIATIONS.

D. E. LEA AND M. H. SALAMAN.*

From the Strangeways Research Laboratory, Cambridge.

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THE object of the experiments described in this paper was to obtain information about the structure of the vaccinia virus particle by a radiological method, using alpha, X-, and gamma rays.

A good deal of information has accumulated concerning the chemical characters of the vaccinial elementary body. Workers here and in America, using relatively large quantities of purified virus, have been able to show the presence of protein, carbohydrate, lipoid, and thymonucleic acid in the elementary body (Hughes, Parker, and Rivers, 1935; McFarlane, Macfarlane, Amies, and Eagles, 1939). Small amounts of two enzymes, phosphatase and catalase (Macfarlane and Salaman, 1938), have been found associated with these purified virus suspensions, also a copper compound and a flavine (Hoagland, Smadel, and Rivers, 1941*a* and *b*); but it is not yet certain that these are essential constituents of the virus. If they are, their function is quite obscure.

The physical characters of the virus have also been examined in detail (McFarlane, Macfarlane, Amies, and Eagles, 1939). Consideration of its homogeneity of size, its behaviour in solutions of sucrose or of electrolytes, and other properties, led McFarlane and his co-workers to put forward the hypothesis that the vaccinial elementary body has a rigid or semi-rigid structure akin to that of a giant molecule; and they suggested that it might be composed of "bricks" of protein cemented together by nucleic acid and lipoid, and surrounded by a layer of bound water. However, when the elementary body was disintegrated by alkali no sign of homogeneity of size among the resulting fragments could be found. No positive evidence of internal structure was obtained.

Microscopic observation of the behaviour of vaccinia inside host cells has shown that the "inclusion bodies" formed have a well-defined structure and pass through a regular cycle of changes (Bland and Robinow, 1939). But no information could, of course, be obtained by this method about the internal structure of the elementary body.

It is largely because of lack of knowledge of the structure of this and other virus particles that it is still possible for those whose work has familiarized them with the biological affinities of viruses to regard them as small unicellular organisms, and for those who have been chiefly concerned with their physical and chemical characters to think of them as giant molecules. The use of radiations is one of the most hopeful methods available for investigating the internal structure of virus particles.

* Of the Cancer Department, St. Bartholomew's Hospital.

In recent years radiation experiments have been of considerable value in genetics. It has been established (see, e.g., Timoféeff-Ressovsky, 1937) that a gene mutation can be produced by a single ionization in the right place. Thus a single ionization in the genetically important matter of the cell may produce an effect which is observable in the cell or is lethal to it. It is very unlikely that a single ionization in the cytoplasm of a cell can produce a detectable effect. Observable effects due to irradiation of the cytoplasm are of course known, but these are due to the cumulative chemical effects of large numbers of ionizations. The effect of a single ionization is primarily to cause chemical change in the molecule in which the ionized atom lies. It is unlikely that the content of the cytoplasm is so nicely balanced that chemical change in one molecule can have important effects on the cell. But the genetic balance may very well be upset by the inactivation or mutation of one gene molecule.

Thus, if in an irradiation experiment we obtain evidence that the observed effect is produced by a single ionization, we may reasonably infer that the effect is being produced by ionization of the genetically active part of the cell. We may then use the radiation experiments to determine the volume of this radiosensitive genetically-active material.

Suppose the volume of the radiosensitive material in the cell is, say, 10^{-5} cubic microns, then a dose of radiation sufficient to produce, say, 1000 ionizations per cubic micron has a chance of 0·01 of producing an ionization in the radiosensitive material. A dose of this size will therefore produce the effect in about 1 per cent. of the cells irradiated. Another similar dose will produce the effect in 1 per cent. of those surviving, and so on. Thus the proportion surviving diminishes in geometrical progression as successive increments of dose are given, and if the logarithm of the surviving fraction is plotted against the dose a straight line is obtained.

Clearly we may work backwards and deduce from the gradient of the survival curve the volume of the radiosensitive material of the cell. It can readily be shown that, using natural logarithms, the dose of radiation required to reduce the logarithm of the surviving fraction by unity is that dose necessary to produce $1/v$ ionizations per unit volume when v = the volume of the radiosensitive material. This we may define as the *inactivation dose*. This calculation assumes that the ionizations are separate. In practice they tend to occur in small clusters, averaging three ionizations per cluster, the clusters being sufficiently compact to act as a single unit. The inactivation dose is thus $3/v$ ionizations per unit volume. A further complicating factor (cp. Lea, 1940*a*) is that, with some radiations, namely soft X-rays, neutrons, and alpha particles, the ionizing particles which dissipate the energy in the irradiated material may produce consecutive clusters so close together that two or more may fall in the sensitive volume at once. This clearly reduces the efficiency *per ionization*, since the two or more clusters produce no more effect than one, and the dose is increased by a factor, F, depending on the size of the sensitive volume or " target " in relation to the mean separation of consecutive clusters along the path of the ionizing particle. For gamma rays, which produce clusters well spaced out along the path of the ionizing particle, F is practically unity.

The application of these principles in a particular case involves two stages. First we must show that the action investigated is of the type in which a

single ionization can cause the observed effect. Then we have to determine the inactivation dose. The characteristics of the single ionization type of reaction have been discussed elsewhere recently (Lea, 1940*a* and *b*), and we shall merely state them without proof.

(1) The survival curve is exponential, i.e. on plotting the logarithm of the proportion surviving against dose we obtain a straight line.

(2) The efficiency of a given dose is independent of whether it is given at high intensity for a short time or is spread over a long time by the use of low intensity or fractionation of dose.

(3) Radiations which produce ionizations close together along the path of the ionizing particle are less effective per ionization than radiations which produce them far apart. This means in practice that alpha rays, neutrons, and soft X-rays are less effective than hard X-rays and gamma rays, and is an important distinction, since a number of actions of radiation not of the single ionization type vary in the opposite direction (e.g. neutrons more effective than X-rays).

When these principles are applied to the study of the inactivation of plant viruses by radiation (Lea, 1940*b*; Lea and Smith, 1940), the three tests indicate that we are here dealing with a single ionization type of action. When the volume of the radiosensitive material is determined from the inactivation dose it is found that it agrees fairly closely with the total size of the virus. Thus the view that the plant virus is a macromolecule or naked gene, which various workers have suggested on other grounds, is borne out by the radiation experiments. Similar methods have been applied to other small viruses. Several varieties of bacteriophage, of diameters between 30 and 50mμ., have been irradiated (Luria and Exner, 1941), and in these also approximately the whole particle is radiosensitive.

In the experiments described in the present paper the same methods have been applied to vaccinia virus, with the object of determining the size and distribution of radiosensitive material in one of the largest viruses. By the application of the argument outlined above it should be possible to decide whether vaccinia is more akin to the smaller viruses or to the bacteria and other unicellular organisms in this respect.

METHODS.

Preparation of Virus Suspensions.

The strain of vaccinia used in these experiments was the same as that used in previous studies by one of us. Originally derived from Lister Institute sheep lymph, it had been through more than 100 serial passages on rabbit's skin before the present work was begun. The method of passage, harvesting, and preparation of pure elementary body suspensions was based on that of Craigie (1932), and has been fully described (Macfarlane and Salaman, 1938). In the present work 3rd day rabbit skin pulp was used, and the resulting elementary body suspensions in M/250 citric acid and disodium phosphate buffer pH 7·1 (the yield of one rabbit in 10 ml.), showed a very high degree of purity as judged by examination by dark-ground illumination. They gave intradermal titres on rabbits of 10^{-7} to 10^{-9}. The suspensions were stored under ether at 0–4° C.

Some of the radiations used in these experiments have a very limited penetration and it is necessary therefore to irradiate the virus in thin films. If a drop of the virus suspension is dried on a coverslip a thin film is obtained, but it is not possible to resuspend the virus. The following method was therefore adopted. A drop of warm 5 per cent. solution of gelatin in distilled water was spread over the surface of a coverslip and dried, the film so obtained having a thickness of less than 5μ. A 1 : 10 dilution of the stock suspension of elementary bodies in the above buffer solution was made, and a measured drop (0·025 ml.) placed on each of a number of gelatin-coated coverslips. The drops were dried at 37° C., yielding deposits of about 7 mm. diameter. One coverslip was irradiated by each of the methods being used, and one kept as control. Irradiated and control coverslips were then dropped into 2·5 ml. volumes of normal saline containing 10 per cent. normal rabbit serum and placed in the incubator for 15–20 minutes, when the gelatin dissolved and the virus was easily resuspended with a capillary pipette. Serial dilutions were then prepared in the same diluent from each suspension. Tests showed that no detectable loss of virus occurred during the drying and resuspension. Volumes of 0·2 ml. of each dilution were injected intradermally on a rabbit's flank. Suspensions of virus treated by each of the three types of radiation, and an untreated control, were titrated on the same animal.

Technique of Irradiation.

For the alpha ray experiments, a source of about 0·1 millicurie of polonium deposited on a silver disc about 1 cm. diameter was placed at a distance of 2 mm. from the virus film. The dose rate at the film was measured by a shallow ionization chamber, the entrance window of which occupied the same position relative to the source as that normally occupied by the virus film. The intensity varied with date as the source decayed ; it was of the order of 1000 r per minute.

The X-ray experiments were made with the aid of a laboratory-built, continuously evacuated tube of the ionic type, which produced nearly pure copper-K radiation, wave-length 1·5 A.U. This tube and the method of measurement have been described elsewhere (Lea, 1941). The intensity was usually about 10,000 r per minute.

For the gamma ray experiments 1 g. of radium was available in five small cylinders, which were disposed symmetrically in a pentagon formation round the virus preparation. The intensity was measured with a small graphite ionization chamber and found to be 128 roentgen per minute.

Titration of the Virus.

For this experiment the usual tenfold dilution series was considered to be too coarse, and threefold dilution was therefore used. With tenfold dilution series, repeated titrations of the same series should give endpoints (taken as the highest dilution giving a just perceptible lesion 5 to 6 days after intradermal injection) rarely differing by more than one place. With threefold dilution series a wider variation is to be expected. Indefinite endpoints, such as $+++0+000$ or $++++00+000$ in place of the definite endpoint

++++ 0000 will be fairly frequent.* Some calculations were made of the degree of variation to be expected, as a guide to the interpretation of the results.

If the virus suspension being titrated is such that the inoculum of highest concentration contains X infective units, then successive inocula in the series will average X, X/3, X/9, X/27 . . . etc. units. It has been shown by Parker (1938) that the observed variation in repeated titrations of vaccinia by intradermal injection in rabbits is best fitted by the hypothesis that one virus particle is capable of producing a detectable lesion. This means that if an inoculum *averages* x infective units, the probability of its *actually* having none, and so giving no lesion, is e^{-x}. Thus the probability of getting the scheme +++ 000 will be—

$$(1-e^{-X}) \cdot (1-e^{-X/3}) \cdot (1-e^{-X/9}) \cdot e^{-X/27} \cdot e^{-X/81} \cdot e^{-X/243}.$$

Choosing an arbitrary value of X one can calculate in this way the probability of getting an endpoint at any given place in the dilution series. Thus with X = 27 we calculate that the probabilities of the 1st, 2nd, 3rd . . . 7th place in the dilution scheme being the endpoint are—

0·000 ; 0·011 ; 0·212 ; 0·365 ; 0·145 ; 0·017 ; 0·001.

When these probabilities are plotted against place in the dilution scheme we get a nearly symmetrical curve the width of which is not significantly changed by changing the value of X. Measuring the width by the root-mean-square deviation from the mean (i.e. standard deviation) we find it to be 0·77 places. The mean endpoint is at a position corresponding to an average of 1·1 infective units in the inoculum.

The seven probabilities listed above add up to 0·75 ; 75 per cent. of experiments therefore should give a definite endpoint. We calculate that 15 per cent. of experiments should give an indefinite endpoint of the type +++ 0 + 000, the remaining 10 per cent. being indefinite endpoints of rarer types. When a definite endpoint is obtained it is of course taken to be the highest dilution giving a positive result. With indefinite endpoints the most satisfactory simple convention is to take the highest concentration giving a negative result.

Out of 40 experimental dilution series analysed only two gave indefinite endpoints instead of the expected ten. This discrepancy is presumably due to a natural bias on the part of the observers in favour of definite endpoints, resulting in a tendency to interpret doubtful lesions accordingly.

Two suspensions were used. Suspension I was used in four experiments and the endpoints of the controls were distributed with a standard deviation of 0·57 places. Suspension II was used for six experiments and the standard deviation of the last five was 0·76. These figures agree with the calculated standard deviation of 0·77 places. In suspension II a considerable (100 ×) reduction in activity occurred soon after its preparation, so that the first experiment was not included in calculating the standard deviation.

It should be noted that the significance of the radiation results does not depend on the purity of the suspensions, nor on the proportion of infective

* + = perceptible lesion. 0 = no perceptible lesion.

to non-infective particles originally present in them. The presence of non-infective material could not alter the ionization in the infective particles in the dry state, and the films were so thin that no correction for absorption of the radiation was necessary.

In plotting the experimental results the displacement of the endpoint of the irradiated virus series compared with the control series on the same animal was used as the measure of the effect of the radiation. The standard deviation to be expected for this displacement is therefore $0{\cdot}77\sqrt{2} = 1{\cdot}09$ places. The standard deviation experimentally obtained, as determined from the deviations between the experimental points and the best straight lines through them, varied between 0·8 and 1·2 in different curves, in close agreement with theory.

RESULTS.

Two series of experiments were made, using separately prepared vaccinial suspensions. The rabbits were examined on the 3rd, 4th, 5th, 6th, and 7th days after inoculation. The control series gave invariably the same endpoint on the 5th and 6th days. The irradiated series sometimes gave a lesion on the 6th day not visible on the 5th. It is possible that the radiation has the effect of retarding the multiplication of such of the virus as is not inactivated. The 6th day endpoint has therefore been used in determining the inactivation doses.

In exhibiting the results graphically we plot as ordinate the number of places by which the endpoint of the irradiated series is displaced relative to the control endpoint on the same animal, against the dose of radiation as abscissa. This is equivalent to plotting the logarithm of the concentration of active virus remaining after irradiation. Fig. 1 shows the curves so obtained for the three radiations; it is based on the experiments made with the second virus suspension. The uncertainties indicated on the graph ($\pm$ 1·09 places) are the theoretical standard deviations. With uncertainties of this magnitude it is, of course, impossible to establish the shape of the inactivation curve with a high degree of accuracy unless a very great number of replications be made. However, it is clear from the graphs that a straight line fits the experimental points satisfactorily. We have checked this by measuring the deviation of each point from the straight line graph and hence calculating the experimental standard deviation from each graph. The standard deviations obtained (see Table I) average 1·05—in good agreement with the theoretically expected 1·09. There is thus no indication that the experimental points deviate more from the straight lines than by the inevitable uncertainties of the titration method.

TABLE I.—*Experimental Standard Deviations of Endpoints.*

	Alpha rays.	X-rays.	Gamma rays.
1st suspension . . .	1·12	1·20	0·95
2nd suspension . . .	1·21	0·99	0·81

Average 1·05.

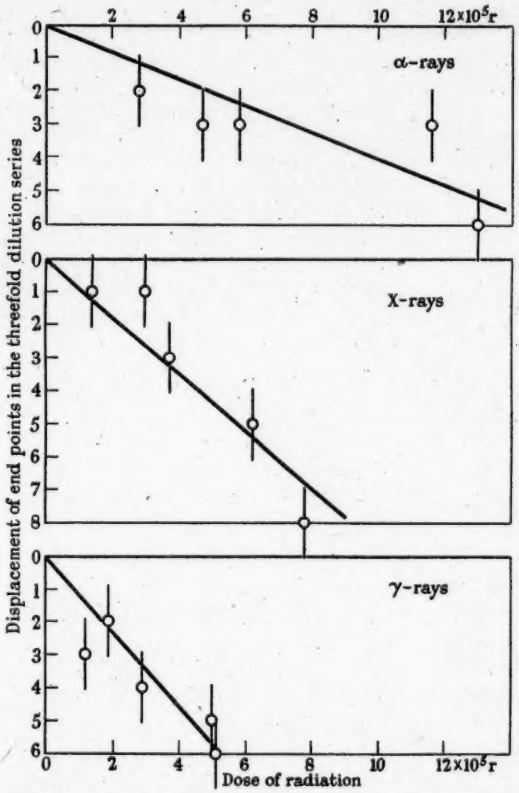

FIG. 1.—The inactivation of vaccinia virus by α-rays, X-rays, and γ-rays.

TABLE II.—*Inactivation Doses.*

	Alpha rays.	X-rays (1·5 A.U.).	Gamma rays.
Experimental :			
Suspension I . .	2·00 ± 0·30 .	1·01 ± 0·18 .	0·85 ± 0·14
Suspension II . .	2·28 ± 0·36 .	1·05 ± 0·11 .	0·79 ± 0·07
Weighted mean . .	2·11 ± 0·24 .	1·04 ± 0·10 .	0·80 ± 0·06
Theoretical :			
(*a*) If whole virus particle is radiosensitive	0·604 .	0·036 .	0·006
(*b*) If inactivation is interpreted as lethal mutation . .	2·11 .	1·12 .	0·80

All × 10^5 roentgen.

The gradients of the straight lines best fitting the experimental points are determined by the least squares method. The dose of each radiation required to cause a one-place shift in endpoint being so determined, the dose for a reduction of concentration by the factor $e = 2{\cdot}718$ is obtained by dividing by $\log_e 3$. This dose we define as the inactivation dose (see Introduction). The inactivation doses for both suspensions used are collected in Table II. The theoretical values given below are described in the following section.

An experiment to test whether the inactivation dose is independent of the intensity at which the radiation is administered was made with X-rays. An exposure of 780 minutes to an intensity of 832 r/min. was made and serial threefold dilutions made and inoculated in quadruplicate. On the same rabbit serial dilutions of the control virus were inoculated in duplicate. The average endpoint displacement produced by this dose of $6{\cdot}48 \times 10^5$ r was 6 places; calculating its standard deviation in the manner described above it was found to be $\pm 0{\cdot}68$ places. The inactivation dose at an intensity of 832 r/min. is thus calculated to be $(0{\cdot}98 \pm 0{\cdot}11) \times 10^5$ roentgen.

The inactivation dose of $(1{\cdot}04 \pm 0{\cdot}10) \times 10^5$ roentgen listed in Table II is derived from experiments made at an X-ray intensity of approximately 10,000 r/min. Thus the inactivation dose is independent of the intensity over a range of 12 : 1.

DISCUSSION.

The Bearing of the Results on the Nature of the Virus.

The experimental results given in the last section satisfy the three conditions set forth at the commencement of this paper, and we infer that the inactivation of vaccinia virus can be caused by a single ionization. To calculate the volume of the radiosensitive material in the elementary body we must convert the doses of radiation, which are measured by the ionization produced in air in terms of the roentgen, into ionizations per unit volume in the irradiated material. The conversion factors are approximately known: 1 roentgen of alpha rays corresponds to 2·63 ionizations per cubic micron, 1 roentgen of X-rays (1·5 A.U.) to 1·30, and 1 roentgen of gamma rays to 1·74 ionizations per cubic micron in tissue of density 1 g. per cm.[3] If we take the density of vaccinia to be 1·26 (McFarlane, Macfarlane, Amies, and Eagles, 1939), these figures are increased in proportion and become 3·32, 1·64, and 2·19 respectively. Since for gamma rays the inactivation dose is 3/v ionizations per unit volume, we deduce that—

$$3/v = 0{\cdot}80 \times 10^5 \times 2{\cdot}19, \text{ whence } v = 1{\cdot}71 \times 10^{-5} \text{ cubic micron.}$$

Taking vaccinia to be a sphere of diameter 0·18 μ, its volume is $3{\cdot}05 \times 10^{-3}$ cubic micron. Evidently only a fraction, 0·0056 of the volume of the virus, is occupied by the radiosensitive material.

The inactivation dose with alpha rays is—

$$2{\cdot}11 \times 10^5 \times 3{\cdot}32 = 7{\cdot}01 \times 10^5 \text{ ionizations per cubic micron.}$$

Since in tissue of this density an alpha particle produces about 5000 ionizations per micron path, the inactivation dose corresponds to about $7{\cdot}01 \times 10^5 / 5 \times 10^3 = 140$ alpha particles crossing each square micron of area, or 1 alpha

particle per area of 0·007 square micron. Thus the radiosensitive material presents an area of at least 0·007 square micron, which is about 1/4 of the cross-sectional area of the vaccinial elementary body. But we have shown that the radiosensitive material occupies only 1/200 of the volume of the elementary body. This small volume and comparatively large area cannot be reconciled with the radiosensitive material being in a compact mass such as a sphere. It may be in the form of a thin *sheet*, or a long thin *filament*, or a *large number* of small units. We have no experimental means of deciding between these three alternatives, but choose the last because on the basis of analogy with other organisms it is possible to develop this hypothesis further.

If we assume, then, that the radiosensitive material of vaccinia consists of N " targets " of average radius r, we may proceed further. The ratio of the inactivation doses of alpha rays and gamma rays is—

$$2{\cdot}11 \times 10^5 \times 3{\cdot}32/0{\cdot}80 \times 10^5 \times 2{\cdot}19 = 4{\cdot}0.$$

This is the quantity we have called F, and is the mean number of ionizations produced in the " target " by an alpha particle which inactivates the virus. This is clearly related to the ratio of the diameter 2 r of a target and the mean separation L of consecutive clusters along the path of the alpha particle. Elsewhere (Lea, 1940*a*) a curve has been given relating F to 2 r/L, and we read off from this curve that $2\,r/L = 5{\cdot}6$. For an alpha particle of the energy used in these experiments producing about 5000 ionizations = 1700 clusters per micron path, $L = 1/1700$ micron and 2 r is thus 3·3 mμ. The volume of a target of diameter 3·3 mμ., if spherical, is $1{\cdot}88 \times 10^{-8}$ cubic micron. Comparing this with the estimate derived for the total volume of all the targets we deduce that their number is $1{\cdot}71 \times 10^{-5}/1{\cdot}88 \times 10^{-8} = 909$.

An argument which has been used elsewhere (Lea, Haines, and Bretscher, 1941) to show that the radiosensitive targets in a vegetative bacterium are probably genes applies equally to vaccinia. This argument runs as follows: A diameter of 3·3 mμ. is not impossibly large for a molecule (molecular weight 14,000). If the targets are regarded as molecules, it is arguable that they must be all different, or mainly so, since it is unlikely that the destruction of one part in nine hundred of any particular constituent would inactivate the virus. Now it is a fair assumption that the destruction of one molecule will not prevent all metabolic processes. Presumably when the irradiated virus is inoculated there is opportunity for the destroyed molecule to be resynthesized; that this does not occur suggests that the virus is unable to manufacture the molecule *ab initio*. Originally therefore it must inherit it. The molecule is capable of being *duplicated* when the virus multiplies, but cannot be *created ab initio*. These are just the properties which current theory attributes to the genes.

We note in passing that the diameter of 3·3 mμ. deduced for the target size in vaccinia is rather close to the diameter of 3·8 mμ. found for the average size of the genes in Drosophila by a similar calculation (Lea, 1940*a*).

Thus our conclusion is that vaccinia has some hundreds of genes of diameter about 3 mμ., their total volume comprising a fraction, 0·0056, of the whole volume of the elementary body. The genes are not present in a compact

mass, but are either dispersed throughout the particle, or are confined to a nucleus of a diameter at least half that of the particle. While undue weight should not be attached to our estimate of the number of genes, we believe that they are certainly multiple.

It is clear that vaccinia differs fundamentally from those of the smallest viruses to which the radiation method has been applied. Many of the plant viruses have been prepared in crystalline or para-crystalline form, and are regarded by many workers as macromolecules. The view has been put forward that these should be regarded from a genetic standpoint as "naked genes." The radiation results are in conformity with this view, showing that their inactivation is of the single ionization type, and that they contain little or no radio-insensitive material. If, then, these viruses are regarded as naked genes, from the same standpoint vaccinia must be regarded as a complex of genes and non-genetic material, similar in this respect to a bacterium or a tissue cell. Whether this distinction will prove to be an absolute one, or whether there is a gradation of complexity as the scale of particle size is ascended, future work, it is hoped, will show.

To make it clear how definitely the radiation results argue against the macromolecule model, we have given in Table II the theoretical inactivation doses to be expected on the two models. The closeness of the agreement between the experimental inactivation doses and the estimates calculated on the lethal mutation model is not, of course, to be taken as proving the adequacy of the method of calculation, since the size and number of the genes have necessarily been arbitrarily chosen to fit the experiments.

SUMMARY.

Experiments are described in which the inactivation of vaccinia by alpha rays, X-rays, and gamma rays is measured by intradermal inoculation of serial dilutions in rabbits. The doses for reduction of the concentration of the active virus by the factor $e^{-1} = 0{\cdot}37$ are for the three radiations 2·11, 1·04, and $0{\cdot}80 \times 10^5$ roentgens respectively. Reasons are given for believing that the inactivation is due to a single ionization in a sensitive constituent of the virus, and for identifying this with the genetically important part of the virus. This sensitive material occupies only about $\frac{1}{2}$ per cent. of the total bulk of the virus particle, in contrast to the small plant viruses and bacteriophages in which it occupies a large part, perhaps the whole, of the particle. Calculations are made concerning the number, size, and distribution, of the genes in the vaccinial particle.

It is concluded that, while previous results of irradiation of the smallest viruses are in conformity with the view that they are "naked genes," vaccinia, from the same standpoint, should be regarded as a complex of genes and genetically-inert material, similar, in this respect, to unicellular organisms and the cells of metazoa.

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REFERENCES.

BLAND, J. O. W., AND ROBINOW, C.—(1939) *J. Path. Bact.*, **48**, 381.
CRAIGIE, J.—(1932) *Brit. J. exp. Path.*, **13**, 259.
HOAGLAND, C. L., WARD, S. M., SMADEL, J. E., AND RIVERS, T. M.—(1941*a*) *J. exp. Med.*, **74**, 69.—(1941*b*) *Ibid.*, **74**, 133.
HUGHES, T. P., PARKER, R. F., AND RIVERS, T. M.—(1935) *Ibid.*, **62**, 349.
LEA, D. E.—(1940*a*) *J. Genet.*, **39**, 181.—(1940*b*) *Nature*, Lond., **146**, 137.—(1941) *Amer. J. Roentgenol.*, **45**, 614.
LEA, D. E., HAINES, R. B., AND BRETSCHER, E.—(1941) *J. Hyg.*, **41**, 1.
LEA, D. E., AND SMITH, K.—(1940) *Parasitology*, **32**, 405.
LURIA, S. E., AND EXNER, F. M.—(1941) *Proc. nat. Acad. Sci., Wash.*, **27**, 370.
MCFARLANE, A. S., MACFARLANE, M. G., AMIES, C. R., AND EAGLES, G. H.—(1939) *Brit. J. exp. Path.*, **20**, 485.
MACFARLANE, M. G., AND SALAMAN, M. H.—(1938) *Ibid.*, **19**, 184.
PARKER, R. F.—(1938) *J. exp. Med.*, **67**, 725.
TIMOFÉEFF-RESSOVSKY, N. W.—(1937) ' Mutationsforschung.' Dresden (Steinkopff).

THE NAGLER REACTION: THE BREAKDOWN OF LIPO-PROTEIN COMPLEXES BY BACTERIAL TOXINS.

E. M. CROOK.

From the Department of Bacteriology, University College Hospital Medical School, London.

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THE reaction described by Nagler (1939), of type A *Cl. welchii* toxin with normal human serum, producing an opalescence or a curd of fatty material, has been used as a measure of exotoxin, for the titration of antitoxin (Nagler, 1941), and as a means of identifying *Cl. welchii* (Hayward, 1941). The reaction is produced by toxins from all of Wilsdon's (1931) four types, and is inhibited by type A antitoxin. Macfarlane, Oakley and Anderson (1941) have shown that a solution of crude lecitho-vitellin (hen egg yolk) reacts similarly but more strongly with the toxin. Nagler identified the reacting factor with Wilsdon's type A factor (the alpha toxin of Glenny *et al.*, 1933), but Hayward found that other Clostridia gave the reaction, which in some cases was specifically inhibited

by antitoxin. In view of its use as a diagnostic test for *Cl. welchii* (Hayward, 1941) and in the estimation of α-toxin (van Heyningen, 1941), a quantitative study both of the specificity and the limitations of the Nagler reaction was undertaken.

MATERIALS.

Toxins.

Preliminary experiments were carried out with a dry toxin of *Cl. welchii* type A, strain SR12, precipitated from a modified Hartley's broth with ammonium sulphate. The M.L.D. was 3 mg. (mouse I.V.). All other tests were made with fresh broth filtrates cleared by centrifugation in order to avoid loss of toxin by adsorption on filters (cf. Robertson and Keppie, 1941) and used within an hour of their preparation.

Media.

Cl. welchii was grown in a 4 per cent. solution of Evans, Sons, Lescher and Webb peptone together with 2 per cent. normal horse serum and 0·3 per cent. glucose (R. S. Roberts, personal communication). Roberts has shown that the concentration of glucose for maximum toxin production varies from strain to strain, a result confirmed by the work of Robertson and Keppie (1941). However, as optimum conditions were not necessary in this work, 0·3 per cent. was chosen as the best single value likely to meet the needs of the majority of strains of *Cl. welchii*. For other species of Clostridia the medium was modified slightly, as shown in Table I, the 4 per cent. E.L.W. peptone being retained as basal constituent. The best results were obtained by avoiding autoclaving the medium at any stage. The peptone solution was adjusted to pH 7·4–7·5 and sterilized by Seitz (E.K.) filtration, distributed in 50 ml. lots in 8 by 1 in. tubes, and freed from air by bringing to the boil in a water bath. The solution was cooled to 45° C. for the addition of serum or glucose and immediately inoculated with 2–3 ml. of vigorously growing 16-hr. meat tube culture. The tubes were incubated in a water bath, which gave a better temperature regulation than the usual incubator and brought the cultures up to their growth temperature very rapidly. The method was successful except with *Cl. oedematiens*, which grew better in the anaërobic jar.

TABLE I.—*Composition of Media. Additions to a Basal Medium of 4 per cent. Evans Lescher Webb Peptone.*

Species.	Normal horse serum per cent.	Glucose per cent.	Incubation times at 37° C. (hours).
Cl. welchii :			
Type A	2	0·3	6 (or 18 at 31°)
Types B and C	2	0·3	24
Type D	..	0·2	70
Cl. oedematiens	1	0·1	50
,, *septique*	2	1·0	30
,, *tetani*	..	1·0	120
,, *botulinum*	2	0·1	120
,, *sordellii*	2	0·2	70
,, *tertium*	..	0·5	70
,, *centrosporogenes*	3	..	50
,, *bifermentans*	2	0·5	50
,, *chauvoei*	1	1·0	50

Cultures.

Strains of *Cl. sporogenes* and *Cl. histolyticum* were obtained from Mr. C. A. McGaughey. The classical type A *Cl. welchii* strains, SR12 and S107, strains of *Cl. septique*, as well as recently isolated strains of *Cl. welchii* and *Cl. oedematiens* were obtained from Dr. Muriel Robertson, whilst the remaining Clostridia were mainly N.C.T.C. strains.

Sera.

In the earlier part of the work individual samples of human serum were used, but later, pooled sera from various individuals of different blood groups and all ages and sexes were used. Animal sera were obtained from Mr. C. A. McGaughey. Antitoxins were all from the Wellcome Physiological Research Laboratories.

Egg-saline.

The yolk was separated from the white of a fresh hen egg and beaten up in 250 mls. of physiological saline. 20 g. kieselguhr were stirred into the turbid yellow solution and the mixture filtered through a chardin paper. This treatment removes a great deal of the lipoidal material present and more is removed when the solution is filtered through a Seitz E.K. filter. The resulting light yellow to bluish opalescent solution is preserved in the ice chest. For turbidimetric measurements, the 1 in 500 solution recommended by van Heyningen (1941) is more suitable, but the stronger solution gives better results with the dilution method.

Turbidity Measurements.

End point method.

This was carried out exactly as described by Nagler, using series of precipitin tubes containing 0·1 ml. toxin dilution and 0·2 ml. human serum or egg-saline. Human serum was incubated overnight (16–18 hrs.) at 37° C. and egg-saline any period over 1 hr., though generally it was more convenient to leave these overnight also. In each case, a standard toxin was run in parallel with the unknowns as well as a blank and an antitoxin control for each specimen. The end point was taken as the dilution giving a turbidity greater than the blank.

Photo-electric method.

This is a modification of the method of van Heyningen (1941), using the Evelyn type photo-electric colorimeter as a turbidimeter. 1 ml. of toxin dilution and 2 ml. of dilute (1 in 500) egg-saline are incubated for 15 mins. in a water bath at 37° C. and the reaction stopped by the addition of 5 ml. of physiological saline containing 1 per cent. sodium citrate. Dilute antitoxin solution, as recommended by van Heyningen, does not combine quickly enough with low concentrations of toxin and the turbidities continue to increase on standing; the inhibiting effect of citrate on the Ca ion necessary for the reaction was perfectly satisfactory. The turbidity is read in the colorimeter as a decrease in deflection, using a Wratten No. 62 (green) filter. This gives an absolute measure of turbidity. The method of applying this to toxin measurement is discussed in the section on kinetics.

REACTION WITH HUMAN SERUM.

Preliminary conditions.

Nagler inactivated all sera by heating to 56° C. for one hour. This was found to have no detectable effect on the end point, and had the disadvantage that heating increased the natural turbidity of the serum and made end-point readings a little less sure.

The ammonium sulphate used in the precipitation of standard dried toxins had no effect on the end point of the reaction. Thus, four samples of *Cl. welchii* type A toxin gave the following end points (in M.L.D.) before and after precipitations with ammonium sulphate: 0·03 and 0·04, 0·01 and 0·01, 0·005 and 0·005, 0·005 and 0·004. In the second case the reaction took place in the presence of the excess ammonium sulphate in the dry toxin.

An attempt was made to speed up the test by using higher temperatures. At 56° C. the tubes with the greatest concentration of toxin showed a turbidity slightly greater than the blank, but later the general increase in the natural turbidity of the serum, noted above, caught up and at the end of 1, 2, and 10 hours all tubes were uniform. At this temperature, inactivation of the toxin appears to be faster than its reaction. At 46° C. the reaction was definitely faster, good turbidities often showing within 2 hours, but results were erratic and the end point did not correlate so well with the M.L.D. ; all later tests were made at 37° C.

Variations of end point with serum.

Nagler noted that with any one toxin the sera varied in sensitivity, the ratio of end points at the limits of the greatest range observed being approx. 2 : 1. This was fully confirmed in the present work, but the range was very much greater, the ratio being approx. 25 : 1 (0·12 to 0·005 M.L.D.). It was at first thought that blood group B sera were very much less sensitive than A or 0, but tests of 27 individual sera with toxin SR12/2A showed no obvious correlation. Table II shows that most end points fall in the range 0·02 to 0·05

TABLE II.—*End Points of Sera.*

End point (M.L.D.)	Number of sera in each group.				
	Group A.	Group B.	Group O.	Group AB.	All groups.
> 0·10	1	..	..	..	1
0·10 – 0·06	2	..	1	..	3
0·05 – 0·04	2	2	4	1	9
0·03 – 0·02	5	..	3	..	8
< 0·02	3	..	2	..	5
Totals	13	2	10	1	26

Toxin No. SR 12/2A.

M.L.D. On the basis of this analysis all sera for testing were pooled. There is no evidence that the age of the serum has any influence on its reactivity, but sera which have become contaminated with bacteria are very much more sensitive, e.g. in one case the end point dropped from 0·16 M.L.D. before to 0·008 M.L.D. after contamination (a 20-fold increase in reactivity). The effect cannot be due to a spurious turbidity produced by multiplying bacteria, since (*a*) in the higher concentrations of toxin a big turbidity appears within two hours—not sufficient time for very great multiplication of bacteria ; (*b*) the extra sensitivity is not reduced on filtering through a Seitz E.K. filter ; and (*c*) the turbidity is completely inhibited by *Cl. welchii* type A antitoxin. There was some increase in pH in contaminated sera, but its effect on the reaction was not studied fully. The presence of haemoglobin in the sera did not seem to affect the reaction in any way, although large concentrations tend to obscure the end point. Nor does the presence of emulsified fatty material in sera collected soon after a meal, but the initial turbidity may similarly obscure the end point.

Degree of turbidity.

Although two sera give identical end points, the degree of turbidity and the variation of turbidity with concentration of toxin may differ markedly. A typical result with five sera is shown in Table III. The first and second have approximately the same end point (0·01 M.L.D.), but whereas the degree

TABLE III.—*Variation in Turbidity.*

No.	M.L.D.	·5.	·4.	·3.	·2.	·16.	·12.	·10.	·08.	·06.	·04.	·02.	·01.	·008.
1	Turbidities	.	.	.	4	4	4	.	4	.	4	3	+	−
2		.	.	.	.	.	.	+	+	+	+	+	±	−
3		.	.	.	.	.	.	3	2	+	+	±	±	±
4		4	4	2	+	.	.	+	+	+	+	±	−	.
5		.	.	.	4	3	+	±	−	−	−	−	.	.

Figures represent number of plusses assigned.

of turbidity in the first is high and falls off abruptly, the second has much smaller turbidities falling off very gradually (the results with higher M.L.D.s would presumably resemble those with serum 4). The difference between ++++ and + is too great to be due to day to day variations in estimation, and in some cases the variations were observed in sera (4 and 5) titrated in parallel. This suggests that there are at least two factors in the reaction of the serum, which may be designated "amount of reactant" and "reactivity of reactant," although their relation is obscure as yet (see p. 46, "Kinetics," below).

Role of Ca ion.

Citrated human plasma was entirely inactive, and fresh human serum is inactivated by the addition of citrate, oxalate, fluoride, and phosphate. The inhibitions are overcome by the addition of calcium chloride equivalent to the inhibitor, plus sufficient to make the solution approximately $M/50$ with respect to Ca ion. This concentration restores the full activity, although smaller concentrations restore it to some degree. Macfarlane, Oakley and Anderson (1941) also found Ca ion to be necessary for the Nagler reaction.

REACTON WITH EGG-SALINE.

The same general considerations apply to egg-saline. Since this reaction is much more rapid than with serum, the effect of temperature is more marked, the inactivating action of the higher temperature on the toxin being of less importance in the shorter time. However, the greater speed makes 37° C. a convenient temperature for routine work. The variation of end point from one sample of egg-saline to another is not nearly so marked as with serum. The limits of the greatest range encountered were in the ratio of 10 : 1 after 16 hrs. at 37° C. Age has a much more pronounced effect on most batches of egg than on most sera. The solution decreases slightly in sensitivity over a period of a fortnight or so and then usually deposits a precipitate which makes further use impossible. Occasionally, however, a batch will remain unaltered

in sensitivity for months, even after the growth of moulds. The reason for this is obscure. The pronounced variation in degree of turbidity observed with sera does not occur with egg-saline, but the " quantity-intensity " effect appears in another way. Thus, a given batch of egg gave with toxin SR12/2A an end point of 0·025 M.L.D. after 1 hr. at 37° C. During the same period very little if any turbidity was observed in serum, even with 0·2 M.L.D. However, at the end of 20 hrs. the end point with egg-saline had decreased to 0·001 M.L.D., whilst that with serum reached 0·005 M.L.D., i.e. the serum had " caught up " somewhat on the egg. This was often observed. Ca ion is also necessary for this reaction.

SERA OF OTHER ANIMALS.

Nagler reported that the only sera, other than man, with which he had some indication of a positive reaction with *Cl. welchii* toxin were one specimen of monkey serum and specimens of fowl sera. The following fowl sera were therefore tested : 2 cockerels—18 and 10 months old ; 2 hens—18 months old, in moult ; 1 hen—12 months old, laying (first season). Table IV shows the

TABLE IV.—*Reaction with Fowl Sera.*

SERA/M.L.D.	1·0.	0·8.	0·6.	0·4.	0·2.	0·1.	0·08.	0·06.	0·04.	0·02.
Human	4	4	4	4	3	+	trace	—	—	—
Cockerel (18 mths.)	3*	2*	+*	—	—	—	—	—	—	—
Cockerel (10 mths.)	2*	+*	—	—	—	—	—	—	—	—
Hen 1	2*	+*	—	—	—	—	—	—	—	—
Hen 2	3*	2*	+*	—	—	—	—	—	—	—
Laying hen	4†	4†	4†	4†	4†	4†	4†	3	±	—

* Precipitate forms, turbidity " granular."
† Flocculation and curd of lipoidal material.
Figures represent number of plusses assigned.

results of tests with toxin SR12/2A. As will be seen, the results are peculiar. The sera of cockerels and of hens in moult gave very little turbidity, and this of an atypical kind, whereas the laying hen gave good turbidities, running out beyond the particular specimen of human serum included for comparison. A further interesting point is that the peculiar flocculation and formation of a curd, always present in the higher toxin ranges with egg-saline, but seldom observed with human serum, is fully developed in the serum of the laying hen. This is interesting in view of the fact that lipoidal materials (lecithovitellins) of the same or similar nature are known to occur both in egg yolk and in the sera of laying hens (Roepke and Hughes, 1935 ; Roepke and Bushnell, 1936 ; Laskowski, 1935, 1936, 1938), and it is attractive to speculate as to whether such materials have any role to play in the reaction with human serum. Nothing seems to be known of the occurrence of such substances in the sera of other animals.

Under the ordinary conditions, no other serum tested (horse, pig, sheep, rabbit) gave a reaction, even with 4 M.L.D. toxin in 0·3 ml., thus confirming Nagler's results. On the other hand, when using horse serum-agar plates (50 per cent. serum) for plating out *Cl. welchii* type A strains, it was noticed by Mr. McGaughey that old colonies (2 to 3 days) were surrounded by a halo of opalescence in the agar. Butler (1941) has also noticed haloes on horse serum-agar and ascribed them to a Nagler reaction. Later it was found that they could be prevented by flooding the plate with antitoxin at the time of inoculation, although colony development was not thus prevented. Similar haloes occur with *Cl. oedematiens*. Thus, horse serum can be Nagler-positive under suitable conditions, and presumably other animal sera also. Whether this lack of reactivity is due to a non-specific inhibitor in the serum or to small amounts of natural antitoxins is not known. The inhibition shows up well when serial dilutions of serum are added to the Nagler reaction with egg. 0·5 M.L.D. of toxin SR12/2A in 0·1 ml. saline was incubated with 0·2 ml. egg-saline and 0·1 ml. of different sera diluted 1/1, 1/10, and 1/100. Human, horse, pig, ox, sheep, and rabbit sera all inhibited the reactions at 1/10 and ox serum at 1/100. The fact that human serum can inhibit the reaction with egg seems to explain the lower reactivity of human serum towards toxin. The results are recorded for their possible bearing on Nagler's negative findings with animal sera.

THE LIPOIDAL MATERIAL.

Nagler made no specific reference to the composition of the material responsible for the turbidity beyond remarking that it was "fat-like." Macfarlane, Oakley and Anderson (1941) apparently assumed that it was a simple fat, for they speak of the opalescence as being due both to " . . . the aggregation of finely divided free fat and to the setting free of fat from compounds, probably lipo-proteins." The following preliminary investigation shows that it is more complex.

50 mg. of toxin SR12/2A in 5 ml. saline were incubated 18 hrs. at 37° C, with 10 ml. pooled human serum, and another batch with 13 ml. of 1/250 egg-saline. The lipoid can be separated to some extent by centrifuging at low speeds (3500 r.p.m.), but a much more satisfactory separation is obtained by 2 hrs. at 14,000 r.p.m., whereby the curd forms a compact layer on the surface and is easily taken off without much contamination by subnatant fluid, and two spins allow the bulk of it to be separated cleanly. By vigorous stirring a homogeneous suspension is made in physiological saline and again separated by centrifugation (2 hrs. at 14,000 r.p.m.). The process is repeated until the material becomes difficult to spin out.

From the resulting purified material, non-polar fat solvents (petroleum ether, benzene, etc.) extract very little, whilst weakly polar solvents (ether, chloroform, ethyl acetate, etc.) have no further effect. Again, as the material has positive protein colour reactions, we must assume that the precipitate contains some protein bound to lipoid or, alternatively, that the lipoid is partly insoluble, of the type found in *Br. melitensis* by Miles and Pirie (1939) and called PL2 by them ; or, both of these may be present. More vigorous

treatment of the complex—with boiling alcohol or cold acetone—causes a visible physical change ; much material goes into solution and the remainder no longer floats but precipitates easily. The material soluble in boiling alcohol gives birefringent crystals and contains phosphorus, i.e. is presumably a phosphatide.

The materials were fractionated by the following scheme. The use of tetralin to separate cerebrosides was first suggested by Page (1930). Each fraction can easily be recovered by pouring the extract into acetone which precipitates lipoids insoluble in it. The acetone residues are returned to the first extract and the small amount of cholesterol, etc., in them included in the acetone-soluble fraction.

"Lipoid"—extract twice with 8 volumes of cold acetone. → Fatty acids and aldehydes. Fats. Cholesterol and its esters, etc.

Insol. in acetone—extract twice with 10 volumes petroleum ether ; pour into acetone. → Lecithin. Cephalin. Sphingomyelin.

Insol. in pet. ether—extract twice with 10 volumes of tetralin ; pour into acetone. → Cerebrosides.

Insol. in tetralin—gives biuret reaction and tests for carbohydrate and the Foulger reaction for desoxypentoses (nucleoprotein present?). Lipoidal complexes may still be present, although extraction with boiling alcohol has no further effect.

TABLE V.—*Composition of "Lipoid."*

Fraction.	Composition—per cent. of whole.	
	Serum (human).	Egg.
Acetone soluble	25	40
Petroleum ether soluble . .	15	10
Tetralin soluble	30	20
"Protein" residue . . .	30	30
Total cholesterol	5–10 of acetone soluble fraction	10–15 of acetone soluble fraction

Table V gives some idea of the relative amounts of the different fractions. The figures represent no more than orders of magnitude, as the amounts available were small and this type of analysis not very accurate. Cholesterol was measured in a micro-colorimeter by the Liebermann reaction. The main difference between egg and serum is the greater preponderance of the acetone-soluble fraction in the egg. This is mostly due, as shown by the lower cholesterol content, to fats which are known to figure prominently among the reserve food material in the yolk.

The most important conclusion is that the precipitated lipoids represent a cross-section of all the lipoids present in either serum or egg yolk, and that no one type is represented to any outstanding degree.

NAGLER-REACTING FACTOR IN TOXIN.

The use of this reaction as a means of estimating alpha toxin depends partly upon the establishment of a parallelism between the Nagler end point and the M.L.D. (mouse I.V.) in type A strains. If, in a series of toxins, prepared from a series of type A strains under varying conditions and by varying treatments, the Nagler end point and the lethality for mice run strictly parallel, there is some ground for believing that the Nagler reaction is caused by the principal lethal factor in type A strains. This evidence is by no means final, since (i) the mouse test can hardly be assumed to have great accuracy when using small numbers of test animals, (ii) there is at least one other lethal factor in type A strains (Weinberg and Guillaumie, 1938 ; Ipsen and Davoli, 1939). However the alpha toxin is probably the principal one, as the work of Ipsen, Llewellyn-Smith and Sordelli (1939), Ipsen (1939), and Ipsen and Davoli (1939) shows that the factor eta occurred to a significant extent only in the toxin of a single strain grown on a particular medium.

Table VI sets out the results for 40 batches of toxin prepared from 13 different type A strains and one type D strain. The conditions and treatment vary considerably. It has already been mentioned that although most toxins were prepared on a single medium, those obtained from Dr. Muriel Robertson were grown on media which varied somewhat from strain to strain in their glucose and serum content. Moreover, the SR12 dry toxin assayed by the photo-electric method was grown on a modified Hartley's broth, whilst the S107 glycerinated toxins were grown on Evans peptone and muscle extract (Macfarlane and Knight, 1941), as was the glucose-treated toxin also.

The M.L.D. (mouse I.V.) for the first 36 batches of toxin set out in Table VI was determined by injecting 0·5 ml. of a series of fairly widely spaced dilutions of toxin, using two mice per dilution and six to ten dilutions. The dose to cause 1/2 or 2/2 deaths was taken as the M.L.D. The fact that no deaths occurred in the higher dilutions and that all the animals receiving the lower dilutions died served as a check on the accuracy of this figure. The toxins assayed by the photo-electric method were checked by a more accurate mouse assay. Five dilutions 20 per cent. apart were used, six mice per dilution, and the dose to cause 50 per cent. death found by interpolation, the standard toxin being assayed by the same method.

The table shows that the correlation between end point and mouse M.L.D. is remarkably good throughout. The range covered is from 0·02 to 0·50 ml. In the half dozen cases where the Nagler test indicated a M.L.D. too large to test, injection of 0·5 ml. of neat filtrate failed to kill. It will be seen that serum or egg may be interchanged at will without affecting the results. The correlation was even more accurate when the photo-electric method was used. The glucose treated toxin was allowed to remain on the laboratory bench exposed to the air for about four weeks, during which time it lost about 2/3 of its activity and became contaminated with moulds, from which it was freed by filtration through a Seitz EK. In spite of this, the Nagler end point still correlated with the mouse M.L.D., in this case done with five dilutions, 20 per cent. apart, four mice per dilution.

TABLE VI.—*Nagler End Points and Mouse M.L.D.*

Strain.	Test materials.	Nagler end point (c.c.). Standard.	Nagler end point (c.c.). Test.	Calculated M.L.D. (c.c.)	M.L.D. Mouse I.V. (c.c.)
Type A, SR 12	S	0·001	0·0002	0·04	0·05 (2/2)
,, ,,	S	0·001	0·0003	0·06	0·05 (1/2)
,, ,,	S	0·001	0·001	0·20	0·25 (2/2)
,, ,,	S	0·001	0·0005	0·10	0·15 (1/2)
,, ,,	E	0·005	0·005	0·20	0·25 (2/2)
,, ,,	E	0·001	0·0005	0·10	0·15 (2/2)
,, ,, (pptd.)	E	0·002	0·005	0·50	0·5 (2/2)
,, SR 12/F	S	0·001	0·0005	0·10	0·1 (2/2)
,, ,,	E	0·005	0·002	0·08	0·08 (1/2)
,, ,,	E	0·001	0·0005	0·10	0·05 (2/2)
,, SR 12/R	E	0·005	0·005	0·20	0·2 (2/2)
,, ,,	E	0·001	0·0005	0·10	0·1 (2/2)
,, ,, (pptd.)	E	0·002	0·01	1·0	..
,, S 107	S	0·001	0·0001	0·02	0·02 (1/2)
,, ,, (pptd.)	E	0·002	0·001	0·10	0·1 (2/2)
,, S 107/R	E	0·005	0·0005	0·02	0·025 (1/2)
,, ,,	E	0·001	0·0001	0·02	0·025 (2/2)
,, JE	S	0·001	0·001	0·20	0·40 (2/2)
,, ,,	S	0·001	0·0005	0·10	0·1 (2/2)
,, ,,	S	0·001	0·002	0·40	0·5 (2/2)
,, ,, (pptd.)	E	0·002	0·005	0·50	0·5 (2/2)
,, *,,	E	0·002	0·0002	0·02	0·015 (1/2)
,, JCC	S	0·001	0·0005	0·10	0·1 (1/2)
,, *,,	E	0·002	0·0005	0·05	0·025 (1/2)
,, *,,	E	0·002	0·0005	0·05	0·05 (2/2)
,, V	S	0·001	0·005	1·0	..
,, *,,	E	0·002	0·002	0·20	0·2 (1/2)
,, *,, (pptd.)	E	0·002	0·001	0·10	0·1 (2/2)
,, *DM	E	0·002	0·001	0·10	0·1 (2/2)
,, *BP	E	0·002	0·002	0·20	0·2 (2/2)
,, *SN	E	0·002	0·0002	0·02	0·025 (2/2)
,, *SV	E	0·002	0·001	0·10	0·05 (1/2)
,, LR	S	0·008	0·04	1·0	..
,, ,,	E	0·0004	0·002	1·0	..
Type D early filtrate	S	0·008	0·08	2·0	..
,, ,, ,,	E	0·0004	0·004	2·0	..
S 107 I (glycerinated)	E	photoelectric method		0·00625	0·0072 (3/6)
,, II (,,)	E	,,	,,	0·0150	0·0158 (3/6)
,, (glucose syrup)	E	,,	,,	0·050	0·052 (2/4)
SR 12 (pptd.)	E	,,	,,	3 mg.	3 mg. (2/4)

Standard (except last four), SR12/2A made up at 5 M.L.D./c.c.

Standard (last four) glycerinated toxin from B. C. J. G. Knight, standardized on 50 per cent. death basis.

F = Variant with flat granular colonies.
R = Variant with rhizoid colonies.
* = Toxin obtained from Dr. Muriel Robertson—her figures for mouse M.L.D.
S = Serum.
E = Egg.

Some further evidence is obtained from a study of the Nagler reaction with *Cl. welchii* types B, C, and D. Robertson and Keppie (1941) and Macfarlane and Knight (1941) have shown that the principal toxic constituent

of type A strains reaches a maximum after about six hours' growth. On the other hand, the epsilon toxin of type D strains requires four or more days to attain full activity (Wilsdon, 1931 ; Bennetts, 1932 ; Dalling and Ross, 1938). The alpha toxin of type D strains, on the other hand, is highest in the early stages of incubation (Dalling, personal communication), as shown by anti-toxin neutralization. Table VII shows the Nagler end points in serum for

TABLE VII.—*Nagler Reaction with* Cl. welchii, *Type B, C and D Strains.*

TYPE/HOURS	6.	29.	51.	108.
A	0·0005	0·001	0·002	>0·1
B	0·001	0·02	0·1	>0·1
C	0·0002	0·0005	0·002	>0·1
D	0·01	0·005	0·01	>0·1

each type at different stages of growth. It is seen that in all cases the reaction is a maximum in the first short period (6 to 10 hours) and thereafter decreases. The type D strain appears to develop rather more slowly, but it, too, has lost most of its Nagler reacting factor in four days.

We may thus conclude that the Nagler factor and the alpha toxin are either identical or are very closely associated in many *Cl. welchii* toxic filtrates and prepared toxins. More conclusive proof must, of course, await the separation of the causative agent in a pure form.

REACTION WITH OTHER CLOSTRIDIA.

When Nagler reported that Clostridia other than *Cl. welchii* gave no reaction, he presumably carried out the tests under the same conditions as he used for *Cl. welchii*, i.e. inoculation of a drop of culture into human serum, with or without the addition of broth and incubation for sixteen hours in the anaerobic jar. Since toxin production on serum is poor in sixteen hours, it was thought that more positive results would be obtained by testing filtrates from cultures grown under conditions known to produce toxins, or good growth with atoxic species. So far it has not been possible to demonstrate Nagler reactions with filtrates from three strains of *Cl. septique*, one of *Cl. histolyticum*, three of *Cl. tetani* (one atoxic and two toxic), and one of *Cl. botulinum* (type unknown). On the other hand, seven other species, set out in Table VIII, gave reactions of varying degrees of intensity. The best *Cl. oedematiens* strains produced reactions as strong as *Cl. welchii* filtrates made under similar culture conditions. It is interesting that such species as *Cl. sporogenes* and *Cl. tertium* should be included among the Nagler positive. In all cases where they were available, the homologous antitoxins completely inhibited the reactions. Heterologous antisera (type A *Cl. welchii* chiefly tested) inhibited not at all or only slightly compared with the homologous antiserum. The toxin of *Cl. chauvoei* was not inhibited by the homologous serum, but it is probable that this antiserum was only anti-bacterial or anti-aggressin. Mason (1936) has shown that the labile *Cl. chauvoei* toxin is not neutralized by anti-bacterial serum, nor is an animal protected against *Cl. chauvoei* cultures by an anti-toxic serum. Thus, if we

TABLE VIII.—*Nagler Reaction with Various Clostridia.*

Species.	Strain.	End-point range in egg-saline.	Inhibition by antitoxin.	
			Homologous.	Heterologous.
Cl. welchii	Various	0·005–0·0005	+	—, S
Cl. oedematiens	Cossard	0·05	+	—
,,	Domange	0·1–<0·0005	+	—
,,	Jolly	0·005	+	—, S
,,	140	0·05–0·002	+	—, S
Cl. sordellii	..	0·1–0·02	..	—, S
Cl. chauvoei	..	0·005–0·002	—	—
Cl. sporogenes	..	0·02–0·01	..	—, S
Cl. centrosporogenes	..	0·02	..	—
Cl. tertium	..	0·05–0·02	..	—
Cl. bifermentans	536	>0·1	..	..
,,	2914	0·01	..	S

\+ Complete inhibition.
— No inhibition.
S Slight inhibition.
—, S Slight inhibition not always observed.

postulate that the Nagler reaction with *Cl. chauvoei* is due to a constituent of an exotoxin, the anomalous behaviour is understandable.

Concurrently with this work, Hayward (1941), using Nagler's technique but a more suitable medium, obtained moderately strong Nagler reactions with one strain each of *Cl. sordellii* and *Cl. centrosporogenes* and seven strains of a species closely related to *Cl. bifermentans*. In all cases she found the reaction completely inhibited by *Cl. welchii* type A antitoxin. Hayward used antitoxin from the Lister Institute. The Wellcome Physiological Research Laboratories' product used in the present work, when tested under her conditions, inhibited less with her *Cl. sordellii* strain (personal communication). Further, as noted above, slight inhibitions were observed in many cases with heterologous sera. These may have been sufficient to inhibit a weak reaction completely, i.e. the converse of Hayward's finding is also true, the Nagler reaction with *Cl. welchii* being slightly inhibited by heterologous antisera, e.g. *Cl. septique* antitoxin.

No attempt was made to relate the end point for non-*Cl. welchii* species to the M.L.D. All that was done was to ensure that the culture conditions gave good growth and good toxins in the case of the toxic strains. Nor have the Clostridia been fully explored in this respect. The fact that of two strains of *Cl. bifermentans* tested, one reacted and the other did not, may indicate that there are Nagler-positive strains among the species listed as failing to give a reaction.

In addition to the anaërobes, an aërobic organism, a strain of *Pseudomonas pyocyanea*, which occurred as a contaminant of a batch of egg-saline, was found to give a Nagler reaction in egg-saline. This was not inhibited by *Cl. welchii* type A antitoxin. Nagler also mentions an aërobic organism, a spore bearer, which gave a reaction in serum not inhibited by *Cl. welchii* type A antitoxin.

The conclusion must be that the reaction is not so specific for *Cl. welchii* as Nagler thought, provided adequate conditions allow the cultures to develop their full complement of diffusible products.

KINETICS.

The Nagler reaction may be due either to a simple surface-active substance in the toxin or to enzyme action. The fact that the alpha toxin of *Cl. welchii* is thermolabile and does not diffuse through a cellophane membrane points to the latter alternative, and it is from this point of view that the kinetics have been treated. It is proposed to put on record here only their bare outline. Several unusual aspects, such as the cause of the pronounced induction period, are being investigated further and it is hoped to describe them at a later date. Most of the results have been obtained with egg-saline, but sufficient data are given to indicate that serum shows the same features.

Effect of toxin concentration.

In simple reactions catalysed by enzymes, the initial velocity of the reaction is directly proportional to the concentration of enzyme for low enzyme concentrations, i.e. the velocity-enzyme concentration curves are straight at low and become concave downwards at higher enzyme concentrations. Here the initial velocity is measured as the amount of turbidity formed in 15 mins. when 1 ml. toxin dilution and 2 ml. 1/500 egg-saline are incubated at 37° C. and the mixture then diluted with 5 ml. citrate-saline to stop the reaction. Turbidity is expressed on an arbitrary scale such that—

$$T = 2 - \log D.$$

T : turbidity in arbitrary units.
D : galvanometer deflection in scale divisions.

In the Evelyn type colorimeter the blank is set to a deflection of 100, i.e. $T = 0$, the deflection decreasing as the turbidity increases.

Fig. 1, curve A, shows a typical curve of the amount of turbidity plotted against increasing amounts of toxin. It is seen that the degree of turbidity increases much more slowly than the toxin concentration, i.e. small amounts of toxin produce an inordinately small amount of turbidity. That this is not simply a defect of the measuring instrument is shown by curve B, which is the turbidity produced by 5 M.L.D.s of toxin diluted with saline in various proportions. The straight initial portion of this curve shows that the instrument measures true turbidities in the lower toxin concentrations. The exact shape of the curve alters somewhat from one sample of egg-saline to another, and alters considerably with the age of the sample after the first 5 to 10 days.

For estimating toxin, the turbidity curve for a standard toxin may be determined. This occupies only half an hour. The difficulty of the variation of absolute values of turbidity from one sample of egg-saline to another can be overcome by using van Heyningen's device (1941) and expressing turbidities for each M.L.D. as a ratio to the turbidity at 3 M.L.D. Such a curve of ratios is shown in Fig. 2, and remains constant within 5 per cent. from one sample of egg-saline to another. It covers the limits 1½ to 6 M.L.D. of toxin

per ml. toxin dilution. The first procedure is generally more convenient than the last unless a large number of routine estimations are to be made, since the ratio curve is no longer obeyed after 5 to 10 days. This, of course, is of no moment if a standard curve is prepared each time. In making a measurement of an unknown toxin by the ratio method, 1 ml. of a suitable dilution, found

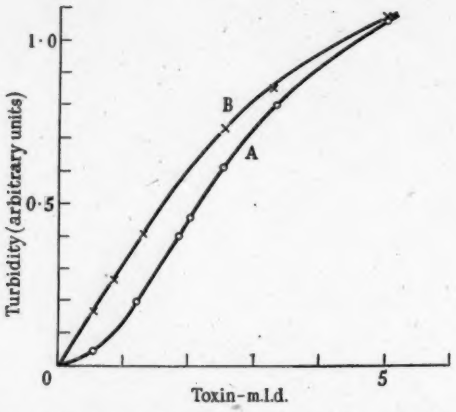

FIG. 1.—*Concentration-turbidity curve :* Curve A :—1 ml. of saline containing various M.L.D.s of toxin and 2 ml. of egg-saline incubated 15 mins. at 37° C. and the reaction stopped by the addition of 5 ml. of citrate-saline. Turbidities expressed on an arbitrary scale. Curve B :—The turbidity corresponding to 5 M.L.D. diluted with saline in varying proportions.

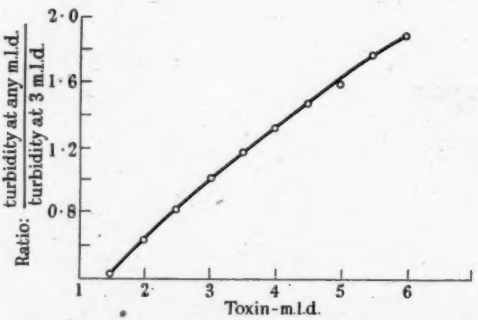

FIG. 2.—Ratio curve : Shows the ratio, turbidity at any M.L.D./turbidity at 3 M.L.D., for amounts of toxin between 1½ and 6 M.L.D.s. Turbidities expressed on an arbitrary scale.

by a preliminary test, and 1 ml. containing 3 M.L.D. of standard toxin are each added to 2 ml. 1/500 egg-saline, incubated 15 mins. at 37° C., the reaction stopped with 5 ml. citrate-saline and the turbidities measured in the photo-electric colorimeter. The ratio (turbidity with unknown)/(turbidity with 3 M.L.D.) is calculated and the number of M.L.D.s in the unknown read from the curve.

Time curves.

When the increase of turbidity with time is examined, some explanation of the peculiar shape of the concentration-turbidity curves is obtained. Fig. 3 shows the increase of turbidity when various amounts of toxin are incubated for various times up to 150 mins. The most prominent feature of the reaction is the pronounced induction period, which increases as the concentration of toxin decreases. As can be seen from curve F, which is almost indistinguishable from curve B, the addition of extra Ca ion to a final concentration of M/50 has no appreciable effect on the induction period although it speeds up

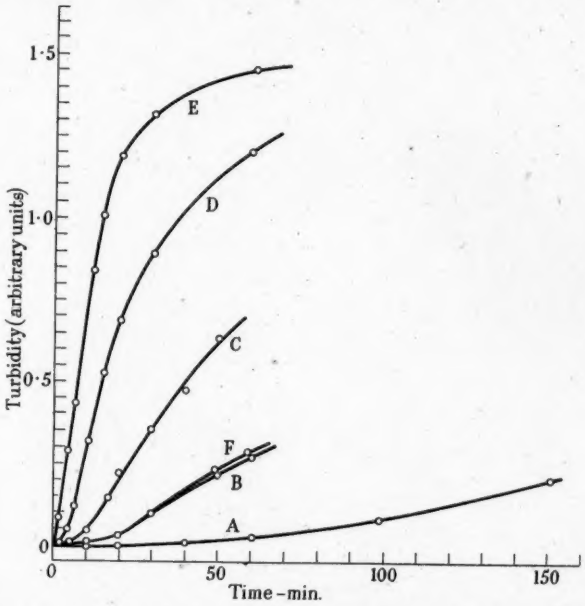

FIG. 3.—Time curves: 1 ml. of saline containing $\frac{1}{2}$ (curve A), $1\frac{1}{2}$ (curve B), 3 (curve C), 5 (curve D), and 10 (curve E) M.L.D.s of toxin incubated with 2 ml. egg-saline at 37° C. for various times and the reaction stopped by the addition of 5 ml. of citrate-saline. Curve F is the same as curve B with $CaCl_2$ added to a final concentration of M/50 during incubation. Turbidities expressed on an arbitrary scale.

the reaction slightly (5 to 10 per cent.). Larger concentrations of Ca ion actually cause some slight inhibition. Thus the induction period is not due to lack of Ca ion.

This induction period, increasing with decreasing concentration of toxin, means that if a given reaction mixture of toxin and egg-saline is diluted, the total amount of lipoidal material formed in a given time is reduced. In simple enzyme reactions with no induction period, the total amount of reaction is not affected by such dilution. It also means that when comparing an unknown toxin with a standard, the time of incubation is critical, for if the unknown

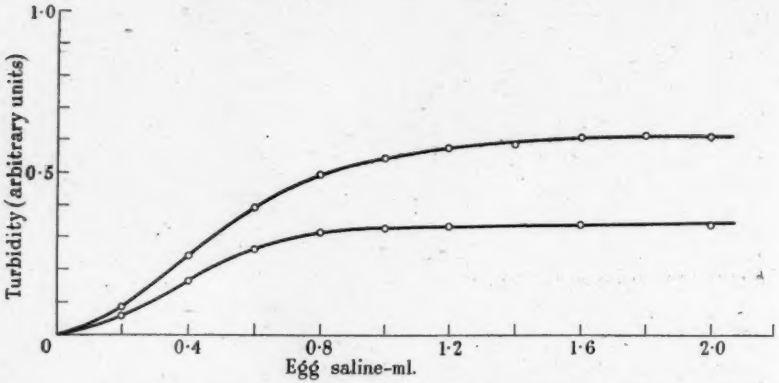

FIG. 4.—Egg-saline concentration curves: 1 ml. of saline containing 3 M.L.D. (lower curve) and 5 M.L.D. (upper curve) of toxin incubated 15 mins. at 37° C. with various amounts of egg-saline, the whole being made up to 3 mls. with plain saline. The reaction was stopped by the addition of 5 mls. of citrate-saline. Turbidities expressed on an arbitrary scale.

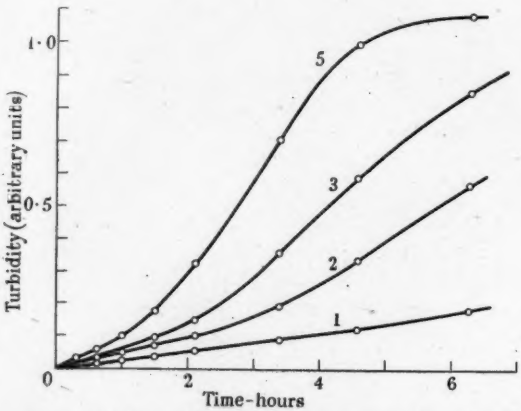

FIG. 5.—Human serum time curves: 3 mls. of saline containing 1, 2, 3 and 5 M.L.D.s of toxin incubated with 6 mls. of human serum at 37° C. for various times. No citrate-saline added. Turbidities expressed on an arbitrary scale.

has a lower concentration of toxin than the standard, its induction period will be longer and vice versa. These have been allowed for in the standard curve for an incubation time of 15 mins., which must therefore be strictly adhered to.

The flattening-out of the curves towards the end of the reaction is to be ascribed to a number of causes, among which are: (i) The instrument effect shown in Fig. 1, curve B; (ii) exhaustion of the substrate so that the enzyme is no longer saturated; (iii) partial inactivation of the toxin at 37° C.; (iv) adsorption of toxin on the precipitated lipoidal material. That the last two

TABLE IX.—*Adsorption of Toxin on Lipoid.*

Saline.		Egg-saline.		Adsorbed.
Initial.	Final.	Initial.	Final.	
10	7	10	0	7
20	14	20	6	8

All magnitudes in M.L.D.s measured by Nagler reaction. "Final" values in egg-saline are for fluid from which lipoid has been separated. Total volume: 4 ml.

factors operate can be seen from Table IX. This sets out the results of an experiment in which toxin was incubated in saline and with egg-saline until the reaction had gone to completion (3½ hrs. at 37° C.). The lipoidal material was spun off and the remaining toxin estimated in each. The toxin lost approximately 30 per cent. of its activity in saline alone, but an extra amount was missing from the egg-saline tubes due to adsorption on the precipitated lipoid. This observation falls into line with those of Myers (1934) and of Frazer and Walsh (1934, 1939) on other toxins and on venoms, which were shown to be adsorbed on fatty emulsions.

Effect of concentration of egg-saline.

Fig. 4 shows that these curves also have a characteristic initial inflection, indicating that the induction period increases with decreasing substrate concentration as well as with decreasing enzyme (toxin) concentration. This is especially the case in those regions where the enzyme is not saturated by the substrate.

It is not possible to give an approximate value for the Michaelis constant (K_m) for the enzyme under these conditions for the nature and concentration of the substrate is unknown. Moreover, the Michaelis constant is defined as the molar concentration of substrate at which the enzyme has half its maximum *initial* velocity. The pronounced induction period makes it impossible to say what the initial velocity may be. In these experiments and those with differing amounts of toxin, the average velocity over the first 15 mins. is measured. Nevertheless, this average velocity reaches half its maximum at about the same concentration of egg-saline for each toxin concentration shown, i.e. we can regard this as a pseudo-constant expressed in terms of 1/500 egg-saline.

Kinetics with serum.

The curves given in Fig. 5 show that the reaction with normal human serum presents all the features of the egg-saline reaction. The major difference is in the scales. The reaction is very much slower, the time scale now being in hours and the turbidity less in the ratio of 1 : 2·7. The "quantity" and "intensity" factors mentioned above appear here as an alteration of induction period and final turbidity. Some samples showed a long induction period but high final turbidity, others shorter induction period and low final turbidity; the alternative combinations were also found. In general, samples with a long induction period tend to show the type of drawn-out end point such as is exhibited by samples 2 and 4, Table III.

DISCUSSION.

The kinetics are sufficiently typical of enzyme kinetics in general that, taken in conjunction with the thermolability and non-diffusability, it may be concluded that the Nagler reaction is due to an enzyme or enzymes. However, the mechanism of the reaction is obscure. Macfarlane and Knight (1941) show that *Cl. welchii* filtrates contain a lecithinase capable of splitting off phosphoryl choline from lecithin, thus liberating an insoluble diglyceride. They think it probable that the turbidity is due to the decomposition of lecithin, which acts as a stabilizing agent for an emulsion of protein and fat. Whether this is the whole story remains to be seen. If we take a lipo-protein association to consist of a " micelle " of lipoid material, polar groups directed towards the outside, on these an adsorbed monomolecular layer of surface-denatured protein, and on this an adsorbed layer of globular protein, there are two sets of conditions under which the association can become unstabilized. Firstly, there may be an attack on the polar groups of the lipoid whereby they are split off (Macfarlane and Knight's case) or reduced in polarity, so that the lipoid separates in an insoluble form, dragging some of the denatured protein with it. Secondly, it is possible that if the stabilizing film of protein is broken down, removed, or otherwise altered the complex may again break up. Lipoproteins are in general susceptible to denaturation and, in this case, the alteration required to produce a turbidity does not have to proceed very far (on either scheme), as indicated by the fact that the analyses of the separated material show appreciable amounts of phospholipides, and protein which is still held with a moderate binding force.

The relation between this power of alpha toxin to disturb lipoid-protein associations and its toxicity is an interesting problem and its investigation should prove fruitful. It may be that toxicity and the power to disturb lipoid-protein associations are two aspects of the same phenomenon, as such associations are known to be widely distributed in living cells. But the filtrates of organisms, such as *Cl. sporogenes*, *Cl. bifermentans* and *Cl. tertium*, are generally regarded as non-toxic. Their Nagler reactions are certainly weak, and, taken on the basis of the ratio for *Cl. welchii* type A, would correspond to M.L.D.s of 1 to 2 mls. They might be regarded as " sub-toxic " rather than atoxic, and the possibility remains that if culture conditions could be found which would increase their Nagler reactivity sufficiently, they might become lethal to mice. Indeed, Weinberg, Nativelle and Prevot (1937) state that under certain conditions *Cl. sporogenes* may produce toxic filtrates. The opposite case is exemplified by strains of *Cl. histolyticum* and *Cl. septique*, both of which produce toxic filtrates which have no Nagler reactivity.

SUMMARY.

The characteristics of the Nagler reaction have been investigated in detail and Nagler's findings substantiated in most cases.

Ca ion is necessary for the reaction; sera other than human can react under favourable conditions; Clostridia other than *Cl. welchii* may give Nagler-positive filtrates, although negative under Nagler's conditions.

For *Cl. welchii* type A, further evidence is presented to show that the reaction is due to the alpha toxin of Glenny.

The lipoidal material separating during the reaction is complex and consists of all types of lipoids and protein.

The kinetics of the reaction in serum and egg-saline are characterized by a pronounced induction period, and are typical of an enzyme or enzymes.

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REFERENCES.

Bennetts, H. W.—(1932) *Commonw. Austral. Coun. sci. industr. Res. Bull.*, p. 57.
Butler, H. M.—(1941) *Med. J. Austral.*, **1**, 33.
Dalling, T., and Ross, H. E.—(1938) *J. comp. Path.*, **51**, 235.
Frazer, A. C., and Walsh, V. G.—(1934) *Brit. med. J.*, i, 424.—(1939) *J. Pharmacol.*, **67**, 476.
Glenny, A. T., Barr, M., Llewellyn-Jones, M., Dalling, T., and Ross, H. E.—(1933) *J. Path. Bact.*, **37**, 53.
Hayward, N. J.—(1941) *Brit. med. J.*, i, 811.
van Heyningen, W. E.—(1941) *Biochem. J.*, **35**, 1246.
Ipsen, J.—(1939) *Quart. Bull. Hlth. Org. L. o. N.*, **8**, 849.
Idem and Davoli, R.—(1939) *Ibid.*, **8**, 833.
Ipsen, J., Llewellyn-Smith, M., and Sordelli, A.—(1939) *Ibid.*, **8**, 797.
Laskowski, M.—(1935*a*) *Biochem. Z.*, **275**, 293.—(1935*b*), *Ibid.*, **278**, 345.—(1936) *Ibid.*, **284**, 318.—(1938) *Biochem. J.*, **32**, 1176.
Macfarlane, M. G., and Knight, B. C. J. G.—(1941) *Ibid.*, **35**, 884.
Macfarlane, R. G., Oakley, C. L., and Anderson, C. G.—(1941) *J. Path. Bact.*, **52**, 99.
Mason, J. H.—(1936) *Onderstepoort J. vet. Sci.*, **7**, 433.
Miles, A. A., and Pirie, N. W.—(1939) *Brit. J. exp. Path.*, **20**, 278.
Myers, G. N.—(1934) *J. Hyg., Camb.*, **34**, 250.
Nagler, F. P. O.—(1939) *Brit. J. exp. Path.*, **20**, 473.—(1941) *J. Path. Bact.*, **52**, 105.
Page, I. H.—(1930) *Biochem. Z.*, **219**, 161.
Robertson, M., and Keppie, J.—(1941) *J. Path. Bact.*, **53**, 95.
Roepke, R. R., and Bushnell, L. D.—(1936) *J. Immunol.*, **30**, 109.
Roepke, R. R., and Hughes, J. S.—(1935) *J. biol. Chem.*, **108**, 79.
Weinberg, M., and Guillaumie, M.—(1938) *Quart. Bull. Hlth. Org. L. o. N.*, **7**, 818.
Weinberg, M., Nativelle, R., and Prévot, A. R.—(1937) 'Les Microbes anaérobies' (Monographies de l'Institute Pasteur), p. 350.
Wilsdon, A. S.—(1931) *Rep. Inst. Anim. Path. Univ. Camb.*, **2**, 53.

ERYTHROCYTE SEDIMENTATION: EXPERIMENTS WITH CONSTANT VOLUMES OF CELLS.

R. B. WHITTINGTON AND A. K. MILLER.

From the Engineering Department, University of Manchester and the Crossley Sanatorium.

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THE red-cell sedimentation test is widely used as a clinical index in pulmonary tuberculosis and in rheumatic diseases. The whole-blood suspensions used in the clinical test involve at least the following five principal variables:

(1) Growth of rouleaux and formation of aggregates.

(2) Red-cell content of the blood—a secondary anaemia being frequently met with in the diseases in which the test finds its widest application.

(3) Viscosity and density of the plasma.

(4) Specific gravity of the cells.

(5) Volume of the individual erythrocytes.

In the pathological conditions mentioned, the first three of these factors commonly show the most marked variation. The clinical test is therefore to some extent a mixed index of the first three factors; but we hope to show decisively, by the experiments to be described, that factor (1) is paramount in producing velocity changes, i.e. that the sedimentation-test is a rough index of the rouleau-aggregation power of the plasma.

The reliability of the maximum sedimentation velocity as an indirect measure of the rouleau-aggregation depends upon the extent to which the other factors mentioned are involved. An increasing anaemia produces increasing sedimentation velocities by reducing the effective viscosity of the suspension; that is, factor (2) operates in the same *sense* as factor (1). On the whole the viscosity of the plasma itself increases with the severity of the pathological condition—that is, factor (3) operates in the *opposite* sense to factor (1). In other words, increasing velocities are, on the whole, associated with increasing viscosities, a phenomenon which would be physically impossible unless the rouleau-aggregation factor were so large as to mask the plasma-viscosity factor entirely.

Now in rouleau-forming, i.e. whole-blood, suspensions, the influence of the red-cell content on the effective viscosity of suspension is not yet quantitatively understood; though in a recent paper (Whittington, 1942) experiments on saline suspensions of washed cells have been described. By the use of erythrocytes from a single subject, saline of constant viscosity and density, and by the elimination of rouleau-formation, the five variables were effectively reduced to one, that of cell-concentration. It was shown that,

given the mean size, specific gravity and number of red-cells, and the viscosity and density of the saline, the actual maximum velocities of the cells could be predicted with reasonable accuracy. In whole-blood suspensions, however, the sedimentation dynamics cannot be said to be understood until the maximum velocities can be predicted from laboratory measurements of the five fundamental quantities listed above.

Meanwhile the results shown in Table I suggest that factor (1) is the most important. It will be seen from these results that the maximum velocity of a highly pathological whole blood suspension may be about 50 times the velocity of a normal whole-blood suspension. Taking, in succession, washed cells (no rouleaux), normal whole-blood (some rouleau-formation and limited aggregation), moderately toxaemic whole-blood (increased rouleau-formation and aggregation), and highly pathological whole-blood, the velocities tabulated are roughly in the ratios 2 : 8 : 40 : 400.

TABLE I.—*Typical Maximum Velocities.*

Description.	Maximum velocity. (cm./sec.).	Packed cell volume per cent.		Dispersion fluid.	
		In suspension.	In the whole blood.	Type.	Absolute viscosity at 20° C. (centipoises).
Washed cells	$2{\cdot}1 \times 10^{-5}$	30	..	Normal saline	1·07
Normal whole blood	$7{\cdot}6 \times 10^{-5}$	35*	44	Citrated plasma	1·60
Whole blood, moderate toxaemia	$3{\cdot}6 \times 10^{-4}$	34*	42	Ditto	1·63
Whole blood marked toxaemia	$3{\cdot}7 \times 10^{-3}$	29*	37	,,	1·84

* Diluted with sodium citrate solution.

EXPERIMENTS.

Experiments with constant volumes of red cells were suggested by Day (1939), but not performed by him. Case 1 was a normal control, while the other three were cases of pulmonary tuberculosis of differing activity, that in Case 2 being by far the most marked. Five suspensions were made from each blood, each suspension containing approximately 23 per cent. of erythrocytes. The cells were pipetted, the percentages not being subsequently checked by centrifuging. The plasma content was varied, the remainder of the dispersion fluid being made up with 3·8 per cent. sodium citrate in normal saline. Each of the twenty suspensions was allowed to sediment in Standard Westergren pipettes, the maximum velocities being obtained as shown in Fig. 1 (sedimentation curves for Case 2).

The approximate percentage of pure plasma in each dispersion fluid was computed ; and a subsidiary experiment having revealed a linear relationship between the viscosity and the percentage of pure plasma, the velocities were approximately corrected for viscosity changes. Thus, increasing the plasma content increases the velocity by increasing the agglutination ; but the same action increases the viscosity (approximately $0{\cdot}10 \times 10^{-2}$ c.g.s. increase in kinematic viscosity for 14 per cent. increase in pure plasma). This means that the velocity increases are partially masked, and should be increased by the correction factor K established in Table II. This is necessarily only

approximate, the mean density of the fluid, and the value of $\frac{\mu_s}{\mu}$ from the Hess (1920) formula being used as for washed cells. Hess's formula is—

$$\frac{\mu_s}{\mu} = \frac{1}{1 - a\theta}$$

(where θ is the percentage of cells in suspension,
,, a is a coefficient varying from 2·25 ($\theta = 0{\cdot}10$) to 1·15 ($\theta = 0{\cdot}79$)).

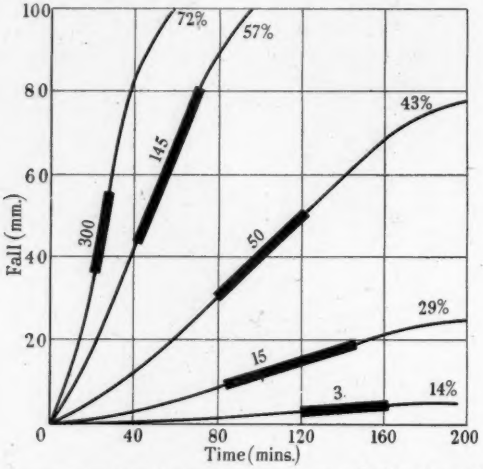

FIG. 1.—Sedimentation curves for Case 2, showing maximum velocities (mm./100 mins.) and plasma percentages.

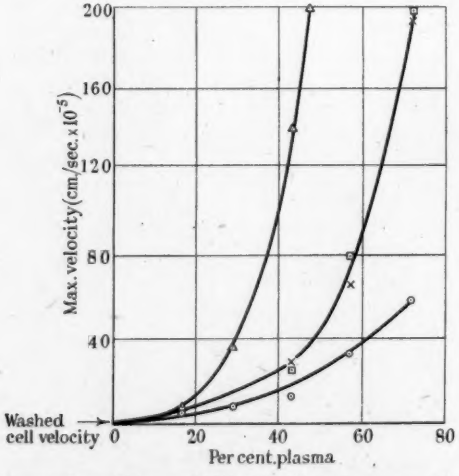

FIG. 2.—Variation of corrected maximum velocity with the percentages of plasma. ⊙ = Case 1, △ = Case 2, □ = Case 3, × = Case 4.

The corrected curves for maximum velocity are shown in Fig. 2. We have now as nearly as possible reduced all the variables to that of plasma percentage. The agglutinative property of the dispersion fluid is reduced as the plasma is reduced ; and the different agglutinative powers of the different plasmas are clearly reflected in the different velocities. It is interesting to note that the curves may be produced backwards to meet the line of zero plasma approximately in the point representing the velocity of washed cells in a fluid of viscosity and density equal to that of the saline citrate. It appears that these curves afford direct evidence that the variations in the sedimentation velocities of whole-blood suspensions are principally due to the agglutinative power of the plasma, the other variables involved being irrelevant.

TABLE II.—*Viscosity Corrections "K."*

Per cent. plasma "p."	0·007 p.	(1·2 + 0·007 p) × 10^{-2} "ν" c.g.s.	$\nu \times 1{\cdot}03$ "μ" c.g.s.	$\mu \times 1{\cdot}9$ "μ_s" c.g.s.	$\left[\frac{\mu_s}{2{\cdot}28 \times 10^{-2}}\right]^2$ "K."
14	0·098	1·30 × 10^{-2}	1·34 × 10^{-2}	2·54 × 10^{-2}	1·24
29	0·203	1·40 ,,	1·44 ,,	2·74 ,,	1·44
43	0·301	1·50 ,,	1·55 ,,	2·94 ,,	1·66
57	0·399	1·60 ,,	1·65 ,,	3·13 ,,	1·89
72	0·504	1·70 ,,	1·75 ,,	3·32 ,,	2·12

$\nu = \mu/\rho$ = kinematic viscosity-coefficient of fluid.
μ = absolute viscosity-coefficient of fluid (1·2 × 10^{-2} at 0 per cent. plasma).
ρ = density of fluid (1·03).
μ_s = effective viscosity of suspension (1·9μ, or 2·28 × 10^{-2} at 0 per cent. plasma).

Maximum Velocities.

Per cent. plasma.	I. Measured.	I. Corrected.	II. Measured.	II. Corrected.	III. Measured.	III. Corrected.	IV. Measured.	IV. Corrected.
	cm./sec.*		cm./sec.*		cm./sec.*		cm./sec.*	
14	4·0	5·0	5·0	6·2	5·2	6·4	6·2	7·7
29	5·3	7·6	25	36	8·0	12	9·8	14
43	6·7	11	83	138	15	25	16	27
57	17	32	242	457	42	79	35	66
72	28	59	500	1060	93	197	90	191

* All velocities to be multiplied by 10^{-5}.

Corresponding washed-cell velocity in fluid of equal viscosity = 1·47 × 10^{-5} cm./sec.

DISCUSSION.

Since the velocity variations are predominantly due to changes in the rouleau-aggregation properties of the plasma, the question arises whether these agglutination changes have corresponding variants in any of the simpler physical properties of the plasma. Obvious possibilities are specific gravity, viscosity, surface tension and electrical conductivity.

Variations in plasma specific gravity are small, the factor of greatest influence being the total protein content. Fraser and Rennie (1941) found little or no correspondence between the total plasma proteins and the erythrocyte sedimentation rate in 220 cases. Gram and Norgaard (1923) found a

slight fall in the electrical conductivity of plasma in cases of pulmonary infections, but were unable to correlate these changes with either haemoptysis, positive sputa, pneumothorax or pleurisy. So far as we are aware, no attempt has been made to investigate the behaviour of the surface tension of blood-plasma.

Using citrated plasma, we found that on the whole the viscosity rose with the maximum velocity, as previously observed by Fahraeus (1921); but there was an indifferent degree of correlation. The concentration of citrate rises as the packed cell-volume of the whole-blood falls; and the estimation of the viscosity of the pure plasma is necessarily approximate, since the division of the citrate between cells and plasma is not known. However, Fahraeus (1921) and Fraser and Rennie (1941) are in agreement that the three main protein fractions exert different influences upon the sedimentation rates. In ascending order of agglutination titre, Fahraeus places albumin lowest, then globulin and fibrinogen. Fraser and Rennie find that the E.S.R. (erythrocyte sedimentation rate) falls as the albumin rises; that there is a rough *direct* relation between E.S.R. and globulin; and a not very marked direct relation between fibrinogen and E.S.R. Clearly the three components all affect agglutination differently; and consequently one cannot expect that any *single* physical property of the plasma should serve as an agglutinative index. In the case of viscosity, for example, though as far as we are aware this has not yet been studied, it is inconceivable that the protein fractions should all exert the same relative influence on the plasma viscosity as on the agglutination.

SUMMARY.

The maximum sedimentation velocity is a rough indirect measure of the agglutinating properties of the plasma, the other variables involved—notably the red cell content and the plasma viscosity—being irrelevant and leading to further inaccuracies.

When agglutinating suspensions are progressively diluted with saline citrate, the velocities ultimately agree roughly with the theoretical washed-cell velocities.

It appears improbable that any one simple physical property of the plasma can be used as an agglutinative index, on account of the plasma-protein complex.

The authors are indebted to Prof. S. L. Baker, of the Department of Pathology in the University of Manchester, for his kind encouragement and advice.

REFERENCES.

Day, G.—(1939) *Tubercle*, **20**, 364.
Fahraeus, R.—(1921) *Acta med. scand.*, **55**, 3.
Fraser, T. N., and Rennie, J. B.—(1941) *Brit. J. exp. Path.*, **22**, 81.
Gram, J. C., and Norgaard, A.—(1923) *J. biol. Chem.*, **56**, 429.
Hess, W. R.—(1920) *Kolloidzschr.*, **27**, 1.
Whittington, R. B.—(1942) *Phil. Mag.*, **33**, 68.